DEVELOPMENT AND CHARACTERIZATION OF AN IMMOBILIZED LYMPHOKINE MEMBRANE

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ABSTRACT

Many soluble growth factors stimulate cells by binding to specific receptors expressed on the surface of these cells. After binding, the receptor-ligand complex will migrate along the cell surface to sites where the complexes are internalized. Through intracellular processes, the growth factors are then sorted and digested, and the receptor may also be destroyed or it may be recycled back to the cell surface. It is not known which parts of this process, from binding through internalization, are required for the growth factor to be able to generate its signal to the cell. This investigation was directed toward discovering whether just the binding of a growth factor that is usually internalized is sufficient to cause signalling. The method used to test this hypothesis was to covalently couple a growth factor to an insoluble support and then incubate this matrix with cells sensitive to the growth factor.

If it could be shown that an immobilized growth factor is capable of transducing its signal to sensitive cells, the development of such as system would be of great value. Growth factors are often recombinant proteins which are quite expensive, and immobilized system could preserve the factor for repeated use in *in vitro* systems and reduce expense. An immobilized growth factor system also offers the advantage of being an affinity matrix for cells which express the specific growth factor receptor. Therefore the matrix could specifically adsorb a subset of cells from a mixed population, and at the same time promote the growth or stimulation of this subset. In addition, since the immobilization of the growth factor will prevent internalization, a regulatory pathway of the sensitive cells will be overturned. It is not known how this upset will effect the cellular response, but it is possible that cells could present reactions to the immobilized growth factor that are not seen with the soluble factor. This would introduce a new method of manipulating cellular behavior.

To pursue this investigation, it was decided to create a model system using the growth factor and lymphokine interleukin-2 (IL-2). This choice was made because IL-2 is a well characterized molecule that is commercially available; it is a potent growth factor for a variety of cell types; it is active via an IL-2 specific receptor and is quickly internalized and digested by IL-2 responsive cells. It is also a clinically relevant molecule associated with therapeutic applications. The IL-2 indicator cell line CTLL-2 was chosen for the model system because it is strongly responsive to IL-2 and dependent on IL-2 for both growth and viability.

An immobilized IL-2 membrane was prepared using a polystyrene based matrix and glutaraldehyde for coupling. Leakage of non-covalently bound IL-2 from the membrane was characterized and shown to be insignificant. Incubation of CTLL-2 cells with the immobilized IL-2 membrane for 36 hours did not cause proliferation of the cells, but the

cells remained viable while on top of the membrane; whereas control cultures incubated on top of an immobilized albumin membrane died.

Further characterizations of the behavior of CTLL-2 cells incubated with the immobilized IL-2 membrane were performed using propidium iodide staining for cell cycle analysis and immuno-fluorescent staining of the IL-2 receptor α chain, an IL-2 induced surface protein. These studies indicated that the immobilized IL-2 did not cause the CTLL-2 cells to be arrested or blocked in their cell cycle, nor did it cause IL-2 receptor α chain expression as soluble IL-2 does. Experiments using Mitomycin C treated cells demonstrated that non-proliferating CTLL-2 cells still require IL-2 to remain viable. Studies were also performed to characterize how cells incubating with immobilized IL-2 will respond to soluble IL-2. The result was that immobilized IL-2 did not cause any abnormalities in their response to soluble IL-2, even though the immobilized IL-2 preserved their viability in a novel fashion.

To gain additional insight into how the immobilized IL-2 membrane affects IL-2 responsive cells, a mathematical model was constructed to predict IL-2 receptor level expression on the surface of these cells. For the case of cells incubating with soluble IL-2, the model predicted behavior which has been observed in this and other studies. When applied to the case of cells incubating with immobilized IL-2, the model parameters had to be modified to predict the observed behavior of the cells. These modifications indicated that the number of active IL-2 molecules present on the surface of the immobilized IL-2 membrane was about 10% of the total amount bound, and that the responding cells decrease their externalization rate of IL-2 receptors while they are incubating on the membrane.

These results demonstrate that an immobilized IL-2 membrane is an effective and non-toxic means of preserving the viability of the IL-2 dependent cell line, CTLL-2. Further applications of the immobilized IL-2 membrane may prove useful for the culture of IL-2 dependent cells.

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CHAPTER 1

1.1. Introduction to Growth Factors and Hormones

Soluble messenger molecules, such as growth factors and hormones, are important elements in regulating the growth and function of cells in mammalian tissues. Hormones like insulin and glucagon are central for carbohydrate metabolism and liver cell function (1); gastrin, secretin, and cholecystokinin are important for digestion; histamine and prostaglandins are potent modifiers of the vasculature; and luteinizing hormone and follicle stimulating hormone exert control over reproductive function, to give just a few examples (2). Some growth factors would include the interleukins and colony stimulating factors which are involved in haematopoeitic growth and differentiation, platelet derived growth factor, transforming growth factors, fibroblast growth factors, and others, for stimulation of endothelial and epithelial tissues; neural growth factor and hepatocyte growth factor cause proliferative responses in specialized organs; and there are many more. In general, these signalling molecules are responsible for controlling the growth and differentiation of tissues, governing the synthesis and secretion of cell products, and regulating the composition of body fluids. These molecules are a chemical form of communication for an organism seeking to coordinate is behavior among different tissue types (3).

The title 'growth factor' is sometimes misleading since a single factor can often stimulate growth in one type of tissue, impede growth in another, and cause altered function in a third in an effort to elicit the optimal response to a given challenge to the organism. This understanding of the basic function of messenger molecules, also called cytokines, has evolved over the last 40 years as factors which were originally identified by their ability to promote growth in some cell types have been found to be much more pleiotropic, more wide ranging in their activities.

1.2. Growth factors and Hormones in Therapy

Along with this recognition of the multiple activities of communication factors has come an appreciation for their potential role in therapeutic applications. While the value of using insulin, human growth hormone, and estrogens has become established; other signalling compounds are also gaining recognition as useful therapeutic tools. α and β interferon have been effectively applied to battle viral infections and hairy cell leukemia, and interleukins and colony stimulating factors are useful for stimulating immune cell growth and overcoming compromised immunity. The IL-1 receptor antagonist can help to block the onset of shock and the acute phase response of the liver, and many different cytokines are being investigated for use in wound healing, cancer, and for ameliorating neural cell damage.

These factors really offer a new approach to therapy as the body's own messenger systems are being directed not by reflex or by metabolism but by the mind of a therapist. Thus natural response mechanisms which evolved to optimize the survival of human species in an era without medicine can now be redirected to take advantage of the surgical and medical support for an individual that is possible in a modern clinic. The danger in this approach is that cytokines interact in many ways with the whole organism. Their use may overcome problems in some areas while causing deleterious effects in others. For this reason, to make best use of this medical technology, it is important to understand how cytokines and what effects they have on all of the cells in the body.

1.3. Basic Function of Signalling Molecules

Communication by extracellular signalling molecules can be broken down into six processes: 1) synthesis and 2) release of the chemical by the signalling cell; 3) transport to the target cell; 4) binding or detection of the signal by the receiving cell; 5) transduction,

where the receiving cell goes through a process recognizing that the signal was received; and 6) response, where the receiving cell adjusts its behavior in some fashion (2). Some hormones, such as steroid based compounds and thyroxine, achieve their signalling effect by passing through the cell membrane of the receiving cells and binding directly to cytosolic or intracellular receptors (4). Other messenger molecules bind to specific receptors on the surface of the receiving cell and the signal is further propagated by second messenger systems that are active in the cytosol (5). This second class of compounds will be the subject of the rest of this review and investigation.

The mechanism by which soluble growth factors which bind to specific surface receptors cause transduction of signal has been elucidated in many respects but much remains unknown. There are two recognized methods by which signal is propagated from the bound growth factor receptor, second messengers and receptor associated enzymes. Second messengers are chemical species produced by the interaction of receptor and ligand that carry the signal into the cytosol and to the nucleus. Some common second messengers are cyclic AMP, calcium ion, and phosphatidyl innositols and innositol phosphates. Receptor associated enzymes are enzymes which become activated, often but not necessarily via G proteins, by the binding of the receptor with its ligand. These enzymes are also commonly tyrosine kinases which phosphorylate different substrates (6). Some features of the fate of the growth factor - receptor complex after binding has taken place have also been discovered. After binding, the receptor-ligand complex will migrate along the cell surface to sites where the complexes are internalized. Through intracellular processes, the growth factors elute from their receptors and are then sorted and digested, and the receptors may also be destroyed or they may be recycled back to the cell surface. It is not known which parts of this process, from binding and migration through internalization, are required for the growth factor to be able to generate its signal to the cell (7).

1.4. Immobilization of Growth Factors

This investigation was directed toward addressing this question of whether binding alone of a growth factor by its receptor is sufficient to cause signalling, or whether the processes of free migration or internalization of the receptor-ligand complex are needed. This question can be tested by chemically linking the growth factor to an insoluble substrate, and incubating responsive cells with this matrix. There are many examples of biological systems that do use immobilized ligands for signalling, such as T cells and B cells in their recognition of antigen and MHC. There are also examples of soluble factors naturally being adsorbed to extracellular matrix components: GM-CSF has been shown to cause proliferation and differentiation of bone marrow cells after being adsorbed by the heparan sulfate component of a stromal cell matrix (8). For the cases of hormones, a controversial paper has reported that insulin immobilized to agarose particles is still capable of generating cellular responses that mimic soluble insulin (9), and that epinephrine coupled to glass beads can produce beta-adrenergic responses in heart cells (10). Yet, there has never been a clear demonstration that a soluble growth factor which is ordinarily internalized can mediate its signal when it is immobilized.

If it could be shown that an immobilized growth factor is capable of transducing its signal to sensitive cells, the development of such as system would be of great value. Growth factors are often recombinant proteins which are quite expensive, and immobilized system could preserve the factor for repeated use in *in vitro* systems and reduce expense. An immobilized growth factor system also offers the advantage of being an affinity matrix for cells which express the specific growth factor receptor. Therefore the matrix could specifically adsorb a subset of cells from a mixed population, and at the same time promote the growth or stimulation of this subset. In addition, since the immobilization of the growth factor will prevent internalization, a regulatory pathway of the sensitive cells will be overturned. It is not known how this upset will effect the cellular response, but it is

possible that cells could present reactions to the immobilized growth factor that are not seen with the soluble factor. This would introduce a new method for manipulating cellular behavior.

1.5. Immobilized IL-2

To pursue this investigation, it was decided to create a model system using the growth factor and lymphokine interleukin-2 (IL-2). This choice was made because IL-2 is a well characterized molecule that is commercially available; it is a potent growth factor for a variety of cell types; it is active via an IL-2 specific receptor and is quickly internalized and digested by responsive cells, and because it is a clinically relevant molecule that is used for immune activation and in the treatment of cancer. It has also been shown that IL-2 can be immobilized to an insoluble support and still bind to its receptors on cells (11).

The question of whether internalization of the IL-2 receptor is required for activation has been addressed peripherally in one study (12), but only relatively recently has the subject been explored directly in two others. In the first, the authors found that a monoclonal antibody which prevents internalization of the high affinity IL-2 receptor, but does not prevent binding, also inhibits growth in an IL-2 dependent cell line. This study is not convincing. The antibody which inhibits internalization is also clearly altering the functional properties of the receptor even though binding of IL-2 may still take place. In addition, the inhibition of internalization of the IL-2R by the antibody takes place with a different kinetic profile than the inhibition of growth. This latter observation is evidence that it is not simply the lack of internalization which is responsible for growth inhibition. In the first investigation which directly looks at using covalent coupling of IL-2 to an insoluble support (13), IL-2 immobilized to polystyrene beads demonstrated augmentation of cytotoxic activities in both *in vivo* and *in vitro* systems. However, the immobilized IL-2 matrix used in these studies was not well characterized and the investigation did not

demonstrate that leakage of IL-2 was not taking place. In a follow up study (14), the immobilized IL-2 matrix was extensively characterized and it was discovered that leakage of IL-2 was significant. To overcome this problem, the matrices had to be washed for 6 weeks at 37°C before use. The immobilized IL-2 matrices prepared in this fashion were not found to have any activity. Two aspects of this experiment which are not adequately addressed are that the thermal deactivation which would take place over the 6 weeks of washing was not accounted for in terms of the final available activity, and that the IL-2 was immobilized to a microporous matrix, thus most of the IL-2 will be present in the pores and not available to interact with cells.

These studies point to the need for a direct investigation of the activity of immobilized IL-2 using a non-porous support where leakage of IL-2 is insignificant and the preparation of the support is not likely to deactivate the IL-2. These investigations are presented in the following chapters. The remainder of this chapter will be devoted to a detailed description of the IL-2 - IL-2 receptor system.

1.6. The IL-2 Molecule

Interleukin-2 (IL-2) is a protein product of T cells of 133 amino acids residues that has been ascribed many biological activities. Originally identified and isolated as T cell growth factor (15-17), the lymphokine has been shown to support proliferation and differentiation of both cytotoxic T lymphocytes and T helper cells (15, 18, 19) as well as B lymphocytes (20, 21). IL-2 also enhances the cytotoxicity of cells from the monocyte lineage (22, 23) and it augments the activity of natural killer (NK) cells (24, 25) and antibody dependent cytotoxic cells (26). In addition, IL-2 is an activation and expansion factor for lymphokine activated killer cells (LAK) (27, 28) and for tumor infiltrating lymphocytes (TIL) (29, 30) which are cell types that can specifically lyse tumor cells. Because of the pleiotropic and

potent activities associated with IL-2, the molecule is the subject of much research for both clinical applications and for fundamental insights into the immune system.

Historically, IL-2 has been recognized as the 'help' factor produced by T helper cells. In brief, when T helper cells recognize the antigens displayed on the surface of an antigen presenting cell, their T cell receptors are bound and cross-linked and the T helper cells become activated. With stimulatory signals from the antigen presenting cells that include other lymphokines, the T helper cells express IL-2 receptors and begin to secrete IL-2. The IL-2 acts as an autocrine growth factor for the T helper cells causing these cells to proliferate. The IL-2 also acts as a paracrine growth and activation factor for cytotoxic T cells and for antibody secreting B cells that have also recognized antigen. The formalism is that antigen recognition moves resting T and B cells from the G_0 to the G_1 phases of the cell cycle, and that IL-2 pushes the cells into the S phase. Thus IL-2 is a vital immune activation factor that is necessary to expand T and B cells populations after they have encountered antigen (31). Studies have also shown that IL-2 is a growth factor *in vitro* for the development of NK cells from bone marrow cells where there is no antigen involvement (32) and that thymocytes express the IL-2 receptor in early stages of development (33); so IL-2 also has colony stimulating factor type activities for T cell development. This means that IL-2 acts both as an activation factor for mature immune cells that are responding to an immune challenge, and as a colony stimulating factor for immature cells.

The primary structure of IL-2 is available from complementary DNA sequences and show the protein to have 133 amino acid residues (16) and is presented in Figure 1.1. The gene for IL-2 is present as a single copy for both humans and mice. For humans, the gene is found on chromosome 4 and consists of four coding sequences (34). The actual three dimensional structure of IL-2 has been solved to 3 angstrom resolution from x-ray crystallographic studies (35) and shows that IL-2 is primarily composed of α helices with a disulfide bridge between cys 58 and cys 105. A representation of this structure is

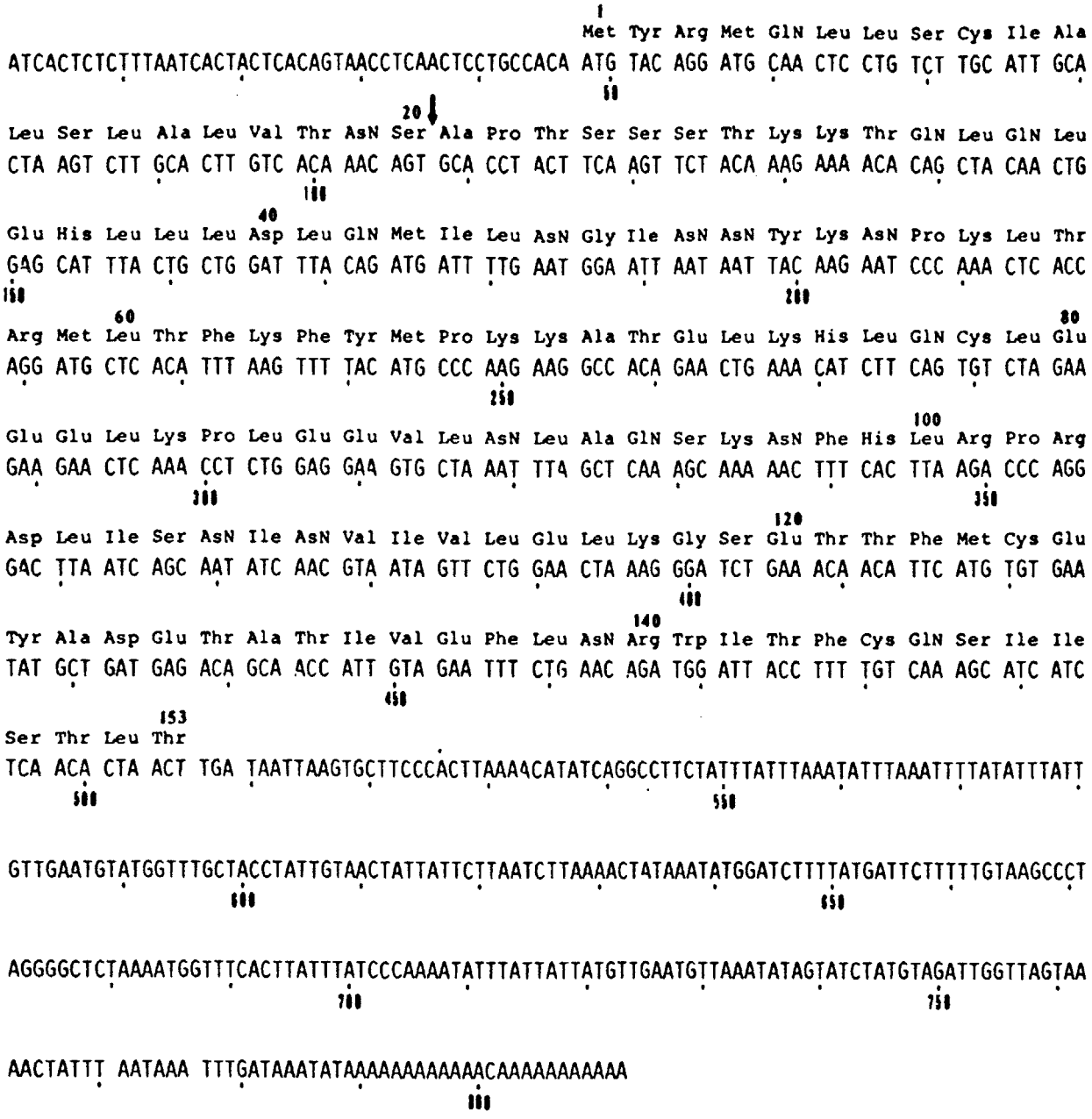

Figure 1.1. The Primary Sequence of IL-2. Taken from Taniguchi, et al. (16).

presented in Figure 1.2. There are six α helical domains that incorporate 89 of the 133 residues present and there are no β pleated-sheet domains. The parts of the IL-2 molecule which make up the binding sites to its receptors have also been elucidated. Using site directed mutagenesis and analysis of binding affinities, studies of the interaction between IL-2 and its p55 receptor (α chain) determined that residues lys 43, lys 35, phe 42, and arg 38 were the major components of the binding site (36). A similar investigation (37) on the interaction between IL-2 and the p70 IL-2 receptor (β chain) suggest that asp 20 and phe 124 could be the relevant residues for this binding site.

1.7. The IL-2 Receptor

IL-2 mediates its biological functions through specific receptors present on the surface of sensitive cells. The first identification of an IL-2 receptor (IL-2R) came through the use of a monoclonal antibody, called anti-Tac, which was reactive with activated T cells and which blocked the binding of IL-2 to these cells. Affinity columns prepared with these antibodies and with IL-2 both specifically bound a protein with a molecular weight of 55 kD from detergent solubilized activated T cells. Subsequent binding analysis proved that these bands were the same molecule and thus the Tac antigen became the first recognized IL-2 receptor (38, 11).

Further characterization of the Tac molecule were performed by Leonard and his colleagues to define the post-translational processing of the molecule (39, 40). By performing pulse-chase experiments and using tunicamycin it was determined that the receptor consisted of a 33 kD peptide precursor after cleavage of the hydrophobic leader sequence. This peptide was co-translationally N-glycosylated to 35 and 37 kD forms, and the 55-60 kD mature protein appears later, suggesting that O-linked glycosylation took place. The 55 kD protein was also shown to be sulfated and it was phosphorylated on a serine residue.

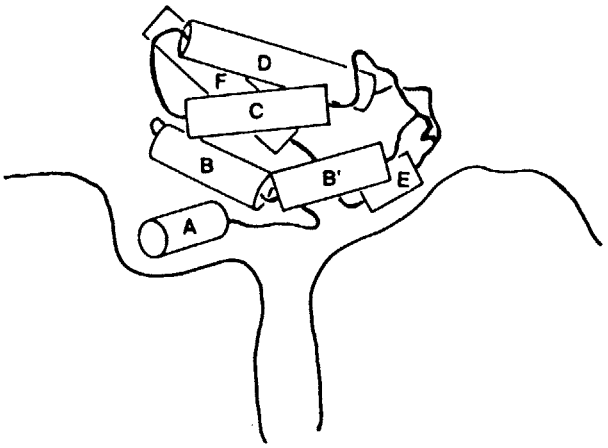

Figure 1.2. Three dimensional Structure of IL-2. Taken from Bradhuber, et al. (35).

Investigations of the Tac molecule continued using radio-labeled anti-Tac and radio-labeled IL-2 to characterize the incidence of the IL-2R in different cell types. A curiosity arose when it seemed that activated T cells and leukemic cells populations displayed 5 to 20 fold more binding sites for anti-Tac than they did for IL-2 (41). This paradox was resolved by the discovery that there were multiple affinity classes of the IL-2 receptor. Scatchard plot analyses for various cell types showed that high affinity receptors (K_d - 30 pM) made up from 5% - 15% of the receptors present while the rest were low affinity receptors (K_d - 30 nM). It is the high affinity receptors which appear to mediate the biological activity of IL-2 since the degree of saturation of these receptors correlate with the proliferative response of the cells (42).

As discussed above for T and B lymphocytes, cells express the IL-2 receptor and become responsive to IL-2 only after they have been "activated" by exposure to antigen. The availability of the anti-Tac antibody provided a tool for kinetic analysis of the appearance of the IL-2 receptor. Lymphocytes stimulated with lectin were found to express peak levels of the IL-2 receptor - Tac molecule after 2-3 days (43). Expression would then decline to low levels after 7-21 days. The presence of IL-2 enhanced the levels of Tac antigen expressed and slowed the decline of receptor expression. The receptors could be reinduced by re-treating with the lectin stimulus. There is also a change in the ratio of high and low affinity receptors during the activation period. During the initial expression phase of the IL-2R the number of Tac molecules might increase tenfold on a stimulated cell whereas the number of high affinity receptors might decline by 20 - 30% (44).

Another interesting phenomenon which occurs with the Tac antigen of the IL-2 receptor is the appearance of a soluble molecule which binds IL-2. After 24 hours of lymphoblast culture with lectin one finds that the IL-2 receptor is released into the culture supernatant. This released receptor accumulates continuously up to day four of culture. Since the total amount of IL-2R (that on the cell surface and that released) is fairly constant after day 3 of

culture, it appears that the shedding of the receptor may be the major mechanism for cell clearance of the activation antigen. The shed antigen has been analyzed and is not only recognized by anti-Tac antibody, it also binds IL-2 efficiently. The released molecule is a glycoprotein with both N- and O- linked carbohydrate and has a molecular weight of about 45 kD. This is 10 kD less than the membrane bound antigen and suggests that the release is by a cleavage mechanism. The reason why the Tac antigen is cleared by this shedding is not well understood. The speculation is that this mechanism reduces the amount of high affinity IL-2 receptors available to the cell and reduces the free IL-2 in the media, down regulating the immune response and making IL-2 itself easier to clear (43, 45). There is evidence to support this idea as sera from some patients suffering from immune disorders have been shown to have very high titers of soluble IL-2R and to be immuno-suppressive *in vitro*. The amount of disease activity correlates with the levels of the soluble IL-2R and the suppressive effects can be partially overcome by adding exogenous IL-2 (46, 47).

The Tac antigen has been cloned and sequenced for both the human and mouse systems (48, 49). The human and mouse p55 IL-2 receptors consist of a 21 amino acid leader sequence, an extracellular portion of 219 and 215 amino acid residues, respectively, a transmembrane portion of 19 residues and a cytoplasmic domain of only 13 amino acid residues. The average homology between mouse and human receptors are 60% in the amino acid sequence and 70% in nucleotide sequence. The cytoplasmic domain is too small to contain any catalytic domain such as is found in the insulin or EGF receptor systems (50). This small cytoplasmic domain, as well as the differences in the number of IL-2 binding sites and anti-Tac binding sites on cells, provided evidence that the Tac antigen alone could not mediate the IL-2 activation signal. Further evidence was being accrued in transfection experiments. Expression of the cloned cDNA of the Tac antigen from activated normal human T cells in mouse L929 (fibroblast) cells and EL4 (lymphoma) cells gave interesting results (34). The L929 cells expressed only low affinity IL-2R and were not responsive to IL-2. The transfected EL4 cells, which ordinarily do not express

Tac, expressed both high and low affinity IL-2 receptors and were responsive to IL-2 in a growth inhibiting fashion. Signal transduction for these EL4 chimeras took place at IL-2 concentrations of 10 pM that would occupy only the high affinity IL-2R.

These results suggested that the Tac antigen required some supplementary molecule to turn it into the high affinity receptor and to transduce signal. The affinity conversion model proposed by Taniguchi (34) hypothesized that the Tac molecule could associate with some other factor ("converter") and to turn it into the high affinity receptor and that this association could only take place if Tac was already bound to IL-2. This condition is based upon the ability of anti-Tac to block IL-2 mediated proliferation even though the relative affinities of association are IL-2 - high affinity receptor > Tac - anti-Tac > IL-2 - low affinity receptor. The converter could be cell type specific, thus explaining why the fibroblast cell line did not express high affinity IL-2 receptors when transfected with Tac while the EL4 lymphoma did. To explore this hypothesis, mutants of the Tac molecule were transfected into the EL4 cell line to determine how the converter and Tac antigen associated (51). The mutations consisted of various alterations underneath the IL-2 binding domain. The result was that all of the mutant proteins formed high affinity receptors and mediated signal, including one that replaced the transmembrane and cytoplasmic domains with those of another protein, and one that extended the extracellular portion by an insertion of 36 residues right above the membrane. This was clear evidence that the converter associates with the Tac protein's IL-2 binding domain and that Tac plays no part in the intracellular propagation of signal.

The search for the converter molecule or other pieces of the high affinity IL-2 receptor continued. The gibbon ape lymphosarcoma cell line MLA 144 is a constituitive producer of IL-2 and has IL-2 receptors of an intermediate affinity (K_d ~ 15 nM). No high affinity receptors were detected and the cells did not react with anti-Tac or with 7G7/B6, another mAb which binds the Tac protein. There was also no mRNA for Tac expressed by MLA 144. Thus the IL-2R molecule on these cells could not be the Tac molecule and isolation of

the receptor yielded a 75 kD protein that was called the β chain (52). Another approach for finding this molecule involved cross-linking IL-2 to its high affinity receptor and then analyzing cell fragments. Using large granular lymphocytes (LGL - the large, dense lymphocytic population from a percoll fractionation) (53), HUT 102B2 cells, normal T cell blasts (54), or EL4 cells (55), a glycoprotein of about 70 kD was isolated. LGL were highly enriched in this receptor compared to peripheral blood mononuclear cells or to T cell populations. Unstimulated LGL and T cells expressed the 70 kD molecule in large excess over the Tac antigen, and when Tac positive cells were removed from a population of peripheral blood lymphocytes, the remaining cells retained all of the IL-2 induced LAK and NK activity of the population.

These findings resolved a longstanding puzzle of how LAK activity could be generated from a cell population when no other stimulus than IL-2 was given and the cells initially had no IL-2 receptors (as defined by anti-Tac). The study (53) also correlated the IL-2 driven response with with the concentration of IL-2 present, with a half maximal point at 500 pM. Prior addition of anti-Tac had no effect on the response of the cells in the first 16 hours. The model that this data suggests is that the β chain is expressed on resting cells that are responsive to IL-2 without antigenic stimulation, making an intermediate affinity IL-2 receptor. Then antigen or IL-2 induces the Tac antigen which combines with the beta chain to form the high affinity IL-2 receptor. Other studies performing cross-linking experiments on other cell lines determined that novel IL-2 binding molecules of 70 or 75 kD were also present on T and B cells and that these proteins were involved in the internalization and transduction of IL-2 mediated signal (56-58). These studies illustrated that the novel receptor could internalize IL-2 and mediate signal in a B cell line which did not express the Tac antigen, and that treating T cell blasts with anti-Tac did not prevent a proliferative response to IL-2, as had been thought, but instead shifted the affinity to IL-2 of the responding cells. All of this data is consistent with the proposal that the Tac protein is not active as a transducing agent of any IL-2 mediated effects but serves as an inducible,

inert low affinity receptor that can combine with the beta chain to form a high affinity receptor and thus heighten the sensitivity of cells to IL-2 (59-60).

The β chain of the high affinity IL-2 receptor has been cloned and its sequence published (61). The mature form of the molecule has 525 amino acid residues with a calculated molecular size of 58,358 daltons. The extracellular region of the receptor contains 214 amino acids and a hydrophobic stretch of 25 amino acids from residues 215 to 239 appear to be the transmembrane domain. The cytoplasmic portion of the receptor is 286 amino acids long and has a consensus sequence for protein kinases in residues 293-295, but not tyrosine kinase activity,

The detection of the β chain of the IL-2 receptor has not completed the definition of this complex or the search for new components. Several pieces of evidence have implicated that a third lymphoid specific component is required for the high affinity and intermediate affinity IL-2 receptors (62). First, the β chain can bind to IL-2 and transduce growth signals when it is expressed on lymphoid cell types, but not when expressed in L929 fibroblast cells (63). Second, the number of β chain molecules expressed on the surface of lymphoid cells is not the same as the number of intermediate affinity IL-2 receptors (64). And third, coexpression of the α and β chains in the L929 cell lines result in high affinity receptors, but they do not respond functionally to IL-2 (48). Once again, some converter molecule that associates with the p75 IL-2 receptor to render it active was suspected and cross-linking experiments uncovered a protein of molecular weight 64 kD that has been identified as the γ chain of the IL-2 receptor (65). Another report in the literature describes a molecule with similar activities but puts the molecular weight at 95-110 kD (66). At present, it is not clear how the γ chain functions. It certainly associates with the β chain, but whether the γ chain a) induces some changes the structure of the β chain to created the intermediate affinity IL-2R or b) binds IL-2 itself is not known. Still, the γ chain has been precipitated with radio-labeled IL-2 from detergent solubilized cells without the use of a

cross-linking agent, and the sequenced structure of the receptor (discussed below) suggest that the chain may bind to IL-2 directly.

The γ chain of the IL-2 receptor has been cloned and the sequence has been published (62). The mature form of the protein consists of 347 amino acid residues with a calculated molecular weight of 39,918 daltons. The membrane spanning region is thought to be a region of 29 hydrophobic residues at positions 255-283 along the protein backbone. The predicted extracellular region (254 residues) includes six potential N- linked glycosylation sites, and the consensus sequences of the cytokine receptor family. The domain also contained a region that suggests a leucine zipper structure. The cytoplasmic domain of 86 amino acid residues is much smaller than that of the β chain but may still have a role in signal transduction. The sequence of amino acids from position 288 to 321 appeared to be homologous to the src homology region 2 which can bind to phosphotyrosine residues of some phosphoproteins.

Finally, the association and dissociation kinetics of IL-2 with the α and β chains of the IL-2 receptor, and with the combined high affinity receptor, have been determined (67). The association of IL-2 to the Tac molecule occurred with a rapid rate: $t_{1/2,\ ass}$ = 4-10 seconds. In contrast, association with and dissociation from the β chain occurred with much slower kinetic rates: $t_{1/2,ass}$ = 40-50 seconds and $t_{1/2,diss}$ = 200-400 minutes. Measurement of IL-2 binding to the high affinity receptor showed an interesting composite of these properties: $t_{1/2,ass}$ = 30-45 seconds and $t_{1/2,diss}$ = 270-300 minutes. These results offer insight into the dynamics of interaction between IL-2 and its receptors and the kind of timescales that are available for the cell to internalize the IL-2 - receptor complex or to transduce signal.

1.8. IL-2 Receptor Expression

The expression of the IL-2R p55 chain by lymphoid derived cells is a dynamic event. This is true not only of B and T cells, as discussed above, but also of monocytes and natural killer cells (NK).

NK cells (68, 69) are are of lymphoid origin and are thought to be a subset of T cells. The specific antigen receptor expressed by NK cells include the γ/δ T cell receptor chains that recognize antigenic determinants in an MHC unrestricted manner (70, 71). NK constitute the major effector cell population capable of mediating MHC unrestricted cytolytic activity and may target tumor, virally infected, and normal cells. IL-2 is a potent growth and differentiation factor for NK cells. Like resting T cells, resting NK cells express little or no high affinity IL-2R but they constituitively express the intermediate IL-2R at low levels. When IL-2 is added at saturating concentrations for these receptors, NK cells proliferated strongly whereas T cells showed no proliferative activity unless monocytes were also present (72). The IL-2 mediated initiation of cell division in the NK cells included induced synthesis of the p55 α chain of the IL-2 receptor. In a separate set of experiments (73) it was shown that an increase in intracellular cAMP levels induced the IL-2R α chain expression in a human NK-like cell line but not in T cells. In addition, treatment with phorbol ester and calcium ionophore, potent inducers of IL-2 α chain expression in T cells, does not induce the α chain in the NK cells.

For monocytes, which are not T cells and have no T cell receptors, the IL-2 β chain is expressed at low levels on resting cells. These cells can be induced to express the α chain when they are treated with either lipopolysaccharide and/or γ interferon. These factors also act synergistically to increase expression of the α chain. IL-2 has not been shown to cause proliferation of activated monocytes that express the high affinity IL-2R but instead to increase effector functions such as peroxide production (74, 75).

It has long been known that B cells can proliferate in response to IL-2 and express IL-2 receptors (76). However, resting human B cells express neither the β or α chain of the IL-2R and do not respond to IL-2 (77). After exposure to antigen, human B cells require 72

hours before they express significant levels of IL-2R (76). In experiments with B cell lines that do constituitively express high and low affinity IL-2R, IL-2 alone was not sufficient to cause proliferation or antibody secretion by the cells (78, 79). Other factors such as IL-5 were required to render the cells responsive to IL-2, and IL-4 could inhibit this action of IL-5.

As discussed above, resting T cells do not express the high affinity IL-2R. After activation with antigen or lectin, these cell transcribe the IL-2R α chain and display the high affinity IL-2R. However, there are differences in receptor expression among T cell subsets. For resting $CD8^+$ cells, the cytotoxic/suppressor phenotype, significant levels of β chain were detected on the cells, but little of the α chain. The case is the opposite for resting $CD4^+$ cells, the helper/inducer phenotype; significant levels of α chain are seen but little of the β chain (77). In addition, treating the $CD8^+$ cells with lectin alone is sufficient to induce expression of IL-2R, whereas the $CD4^+$ cells require accessory cells present, lectin alone is not sufficient for IL-2R induction. A phorbol ester, PMA, could substitute for the accessory cells (80). For both cell types, placing the cells that were induced to express the IL-2R in culture media with added IL-2 caused increase expression of the IL-2R compared to plain media. Thus IL-2 up-regulates its own receptor in normal T cells (81-84).

T cell expression of the IL-2R α chain can also be a function of how the T cells were activated. Two recent studies (85, 86) have shown that engaging the T cell adhesion molecule CD28 during activation with its ligand, B7, a B cell activation antigen, leads to stronger and more prolonged expression of the IL-2R α chain as well as to increased cytokine production by the T cells. Therefore local interactions in the cellular environment also influence IL-2 receptor level expression.

The above studies in this section have all examined the expression of IL-2R *in vitro*, on cell lines or on selected populations of primary cells. There also exists a literature about the expression of the IL-2R *in vivo* (87-91). The results of these studies show that the cell types that respond to IL-2 *in vivo* are usually $ASGM1^+$, and that mature T cell growth is

not accompanied by expression of the high affinity IL-2R in the majority of activated cells (92). There is additional information documenting that manipulations that are effective in altering IL-2R expression levels or the cellular response to IL-2 *in vitro* are not as effective *in vivo*, as would be expected.

1.9. IL-2 Receptor Internalization

From the discussion above, it seems that it is the β chain of the IL-2R, possibly in combination with other proteins, that mediates the IL-2 signal. This is also true of internalization (93). The technique of acid wash at 4° C was used to investigate the internalization parameters of the IL-2R. Studies using this technique with radio-labeled IL-2 and responsive cells showed that greater than 95% of the ligand bound to the cell surface could be removed. Thus by using a pulse of labeled IL-2 and varying the incubation time before the acid wash, a kinetic profile of the internalization of IL-2 can be obtained. Two studies have reported that the half life for internalization of IL-2 by cells expressing the high affinity receptor only is about 10 minutes (94, 95).

Autoradiography, EM, and light microscopy studies were performed tracing the internalized IL-2. Lowenthal (95) reported that the IL-2 follows the classic internalization pathway via coated pits and endosomal channeling to the lysosomes. Fuji (94) reported that the IL-2 may be present in the nucleus of the cell, yet the internalized IL-2 is rapidly degraded and excreted from the cell with a half life of 60-80 minutes. The consensus would be that IL-2 is channeled to the lysosomes after internalization and is not a active cytoplasmic species.

Another study already mentioned (12) found that a monoclonal antibody which prevents internalization of the high affinity IL-2 receptor, but does not prevent binding, also inhibits growth in an IL-2 dependent cell line. The authors conclude, unconvincingly,

that internalization of IL-2 by the high affinity receptor is required for the growth of the cells.

1.10. IL-2 Mediated Signalling

The intracellular pathways that relay the signal that IL-2 has bound to its receptor have yet to be definitively established. The reasons for this has to do with some of the intricacies of lymphocyte activation. A major problem in working out the signal transduction for IL-2 is that IL-2 activation is a second step in the stimulation of T and B cells, occurring after they have already been 'activated' with antigen. Moreover, for T cells, IL-2 activation is often an autocrine process; it is extremely difficult to distinguish between antigen mediated signalling and IL-2 mediated signalling when there is a continuum between the two processes. In addition, as has already been discussed, different cell types respond to IL-2 in different fashions. Yet, though the biochemistry is uncertain, there is much information about some of the molecular biology that follows IL-2 presentation to sensitive cells.

Focusing first on the biochemistry, much of the early work looking at the signal transduction of IL-2 suggested that secondary messenger involved phosphatidyl inositol phosphates (PIP_x) and the activation of phospho-lipase C which cleave PIP_x to produce inositol phosphates (IP_x) and diacylglycerol (DAG) (96, 97). The reason that PI turnover was suspected (and in some cases assumed) was because there was abundant evidence that IL-2 stimulated cells had elevated levels of cytosolic calcium (98, 99) and had turned on protein kinase C and Na^+/H^+ antiport activation (100-102). Diacylglycerol is known to be an activator of PKC and inositol phosphates have been shown to mobilize stores of intracellular calcium; Na^+/H^+ antiport activation is also a product of PI turnover.

The problem was that no study was able to attribute these changes directly to IL-2. Finally, Mills et al. (103) performed sets of experiments that measured production of

diacylglycerol or inositol phosphates from IL-2 sensitive cells which had been washed in IL-2 free media and then pulsed with IL-2. They found that there was no increase in either inositol phosphates or diacylglycerol as a result of the IL-2 pulse. In contrast, these components were greatly elevated after giving the cells phytohemagglutin-P (PHA), a lectin that stimulates the T cell receptor in a manner analogous to antigen. This same group had also shown that IL-2 was not responsible for increases in intracellular calcium (104). Therefore it was established that PI turnover was a result of antigen stimulation and not IL-2. This result has been confirmed in further studies with T (105-110) and B cells (111-114). There are also studies that show that mimicking PI turnover using calcium ionophores to mobilize calcium and phorbol esters to trigger PKC can actually inhibit some IL-2 mediated responses in T lymphocyte cell lines (115, 116).

The most recent investigation on the second messenger system used by IL-2 has implicated the molecule glycosylphophatidylinositol (gly-PI) as the means of signal transduction (117). This compound contains a hydrophobic domain that usually consists of 1,2-dimyristolacyl-glycerol (whereas plain PI usually is composed of arachidonate esters), and a PI unit that is glycosidically linked to a glycan moiety through glucosamine. The authors have shown that myr-DAG is rapidly produced only after IL-2 treatment of an IL-2 dependent cell line and that this response is dose dependent. They also demonstrate similar kinetics in the production of inositol glycans, and they show that gly-PI is rapidly depleted after IL-2 treatment. Though convincing, this report stands alone in the literature and confirmations are needed.

Although the biochemistry of the second messenger system is uncertain, a consensus does exist about some of the molecular biology and later events of IL-2 mediated activation. The most common early events in intramolecular signalling often involve generation of enzymatic activities via protein kinases, and IL-2 is no exception. Examining some of the changes in the IL-2 receptor complex itself after binding to IL-2, it has been demonstrated that the p75 β chain molecule is phosphorylated on a tyrosine residue in response to IL-2

(118-120) and this tyrosine kinase activity co-precipitates with the β chain when cell lysates are treated with an anti-β chain antibody (121). Since the β chain itself does not have tyrosine kinase activity, the conclusion is that a tyrosine kinase physically associates with the β subunit of the IL-2 receptor.

It is now thought that this associated protein tyrosine kinase is the $p56^{lck}$ gene product. The evidence for this supposition is that treatment of T lymphocytes with IL-2 generates $p56^{lck}$ activity, and that the $p56^{lck}$ protein and the IL-2Rβ appear to have a physical association based upon immunoprecipitation studies and site directed mutagenesis of the the proteins (122). There is also a report on IL-2 dependent $p56^{lck}$ activity in NK cells, but in these cells protein kinase C is also stimulatory of the activity (123). Other proteins with molecular weights of 92, 80, 78, 70-75, and 57 kD have been found that are phosphorylated on tyrosine residues in response to IL-2 (119). It has also been shown that IL-2 binding activates a phosphatidylinositol-3-kinase, again by phosphorylating a tyrosine on this enzyme (124). This suggests that some form of phosphatidyl-inositol phosphate might be involved in signal transduction.

The GTP binding protein $p21^{ras}$ is another activity that is turned on by IL-2 (125). This compound is located at the inner surface of the plasma membrane where it binds to GTP and hydrolyzes the nucleotide to GDP. There is much evidence for $p21^{ras}$ involvement in the signalling of cellular growth. In both murine and human T cells, addition of IL-2 caused a rapid and prolonged activation of the $p21^{ras}$ product, and the levels of $p21^{ras}$ expression correlated with the dose of IL-2.

A study to find genes that are stimulated by IL-2 used the technique of creating a cDNA library (126). Poly(A+) RNA from L2 cells that had been stimulated with IL-2 was used to create the library and about 300,000 distinct clones were isolated. The library was screened by differential hybridization using cDNA from poly(A)+ RNA from unstimulated L2 cells. Twenty-one different clones were isolated by this method. The most abundantly IL-2 induced clones were glycolytic enzymes: glyceraldehyde-3-phosphate dehydrogenase

(EC 1.2.1.12), pyruvate kinase (EC 2.7.1.40), triose-phosphate isomerase (EC 5.3.1.1), non-neuronal enolase (4.2.1.11), lactate dehydrogenase (EC 1.1.1.27), and a phosphoglycerate mutase like protein (EC 5.4.2.1). The next most frequently occurring clones were cytoskeletal proteins: vimetin, α-tubulin, β-actin, and γ-actin. Four other clones were identified: elongation factor 2, a transmembrane endoplasmic reticulum glycoprotein ERp99, ribosomal phosphoprotein P1, and a protein which was homologous to the DNA binding protein dbpB, which binds the EGF receptor enhancer. Seven other clones could not be identified.

On a transcriptional level, IL-2 activates nuclear expression of the DNA binding protein NF-κB (127). This eukaryotic transcription factor is regulated at a post-translational level by IL-2 since there is no change in mRNA expression of NF-κB from IL-2 exposure. Interestingly, the enhancer elements for the genes for both IL-2 and IL-2R contain κB binding regions (128). This may explain how IL-2 is able to up-regulate its receptor. TNF-α is also able to activate IL-2R α expression through this κB control region in the receptor gene (129). Additional reports about transcriptional activation from IL-2 examine the genetic elements of the IL-2 induced J-chain protein in a B cell line (130, 131); the control region in the J-chain gene promoter is sequenced and analyzed.

Finally, some information about IL-2 signal transduction can be gleaned from studies on known inhibitors of IL-2. Prostaglandin E2 (PGE_2) is known to inhibit IL-2 and IFN-γ production from T helper cells and is widely viewed as a general immunosuppressive (132-135). PGE_2 functions by increasing cyclic AMP (cAMP) levels through activation of adenylate cyclase (136). A recent study on peripheral blood mononuclear cells (PBL) has elucidated some of the mechanisms of this PGE_2 mediated inhibition of IL-2 activation (137). On the transcriptional level, nuclear run-off assays showed that elevated cAMP inhibited transcription of the IL-2R and IL-2 genes. H-8, an inhibitor of protein kinase A, reversed this inhibitory effect, suggesting that the inhibition is mediated through protein kinase A. Additionally, cAMP decreased the half-life of IL-2 mRNA by more than 50%

and inhibited the tyrosine phosphorylation of a 100 kD protein used as an activation marker. An examination of natural killer cells and large granular lymphocytes (LGL), however, showed that activation of adenylate cyclase actually enhances the level of IL-2R α chain expression (138). It should be remembered that NK and LGL cells do not need antigen priming to respond to IL-2, and cyclic AMP does inhibit NK cell cytolytic functions (139).

1.11. IL-2 in Therapy

One of the primary reasons IL-2 was chosen as a model protein for this investigation on immobilization of a growth factor is that IL-2 is a clinically relevant and interesting molecule. As a potent stimulator and regulatory molecule, IL-2 has been the subject of much therapeutic research since its discovery, culminating in its approval by the Food and Drug Administration (USA) as a treatment for renal cell carcinoma. The clinical research on IL-2 is reviewed in **Appendix A** of this thesis.

1.12. References

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CHAPTER 2

DEVELOPMENT OF AN IMMOBILIZED IL-2 SYSTEM

2.1. Introduction

Interleukin-2 (IL-2) is a pleotropic immune cell factor which has been shown to mediate activity through interaction with a specific, high affinity IL-2 receptor (IL-2R) complex that is expressed on the surface of sensitive cells (Smith, 1988; Zubler *et al.*, 1984; Shiloni *et al.*, 1987; Malkovsky *et al.*, 1987). This receptor is an aggregate of at least three proteins - a low affinity (p55) α subunit (Uchiyama *et al.*, 1981; Leonard *et al.*, 1982) an intermediate affinity (p75) β subunit (Sharon *et al.*, 1986; Robb *et al.*, 1987), and another chain necessary for signal transduction, the (p64) γ chain (Takeshita *et al.*, 1992). Although the biochemical events associated with IL-2 binding to IL-2R and the eventual transduction of signal are becoming clearer, much remains ill-defined (Farrar *et al.*, 1985; Merida *et al.*, 1991; Eardley *et al.*, 1991; Horak *et al.* 1991). While it has been shown that after binding to IL-2, the ligand-receptor complex migrates to internalization sites where the complex is internalized, sorted, and possibly recycled, it is not known whether all of these steps are necessary for communicating the activation signal of IL-2 (Fujii *et al.*, 1986; Lowenthal *et al.*, 1986). Clarification of this issue, whether internalization of the receptor-ligand complex is necessary for IL-2 derived activation, might significantly enhance our ability to manipulate sensitive cells using IL-2. It could also lead to new understanding about the processes of cellular activation and regulation via receptor mediated internalization of growth factors.

The question of whether internalization of the IL-2 receptor complex is required for activation has recently been adressed in several studies. Kumar *et al.* (1987) showed that a monoclonal antibody which prevents internalization of the IL-2 receptor, but does not

prevent binding, also inhibits growth for IL-2 dependent cells. This study does not definitively answer the question. The monoclonal antibody which inhibits internalization is also clearly altering the functional properties of the IL-2 receptor complex, even though binding of IL-2 may still take place. In addition, the inhibition of internalization of the IL-2 recepor complex by the antibody occurs with a different kinetic profile than the inhibition of growth. Crum and Kaplan (1991) prepared IL-2 immobilzed to polystyrene beads and demonstrated augmentation of cytotoxic activities in both *in vivo* and *in vitro* systems. This study did not show that no significant leakage of IL-2 is taking place. Kaplan (1991) has also reported that IL-2 coupled to Sepharose beads did not cause thymidine incorporation in IL-2 sensitive cells in a cellular assay. In this study the immobilized IL-2 matrix was carefully analyzed but leakage was so significant that the matrices could only be used after 6 weeks of washing at 37°C and the thermal inactivation of the immobilized IL-2 was not taken into account. In addition, the matrix used was porous so that the vast majority of the coupled IL-2 would be found in the pores of the material and thus inaccessible to the incubating cells. These important studies point to the need for a direct investigation using IL-2 immobilized to a non-porous matrix where the leakage of IL-2 from the support is insignificant.

In the present report, we have prepared an immobilized IL-2 matrix using plasma activated polystyrene membranes as the support material. The matrix was incubated with the cell line CTLL-2, which is dependent on IL-2 for both growth and viability. This system allows binding to occur between the immobilized IL-2 and the IL-2R expressed on the surface of the IL-2 sensitive cells, but inhibits the free lateral diffusion and internalization of the bound IL-2 - IL-2R complex. This study reveals that immobilized IL-2 can mediate some of the activity of soluble IL-2 and that internalization of the IL-2R after binding may not be required for at least part of the IL-2 mediated effect.

2.2. Materials and Methods

Preparation of Membranes. Immobilized IL-2 membranes were prepared starting from MatTek T2 membranes (MatTek Corp., Ashland, MA). These are nonporous, polystyrene materials which have been gas plasma treated to provide modified surfaces. The surfaces include primary amino groups which were used for the immobilization procedure. The membranes were cut into 1.5 cm diameter circles and washed through an ethanol gradient ranging from 95% to 0% ethanol in water. The circles were then treated with 2% glutaraldehyde for 30 minutes, washed twice in sterile phosphate buffered saline (PBS), pH 7.4, and then treated overnight with 120 ng of human recombinant IL-2 (a gift from DuPont, Wilmington, DE) suspended in 2 ml of PBS. Some membrane preparations used a radiolabeled IL-2 tracer (New England Nuclear, Boston, MA) in the coupling reaction for quantitation of the amount of IL-2 bound. It has been determined through studies using radiolabeled IL-2 that membranes treated with 120 ng in 2 ml of PBS yield final amounts of 4.8 ng or less of immobilized IL-2. After coupling, the membranes were passed through 3 washes in sterile PBS and 3 washes of sterile PBS + 10% (v/v) fetal bovine serum (FBS). Each wash was for a 24 hour period. The membranes were kept at 4 °C for the first 20 hours of the wash but were then moved to a 37 °C incubator for the final four hours. Subsequently, membranes were stored in sterile PBS + 10% FBS at 4°C until use. Membranes could be stored for a month before use with no discernable loss of activity. Immobilized bovine serum albumin (BSA) membranes used as controls were prepared in an identical manner except for the loading of 2 ml of 2% (w/w) BSA in PBS instead of IL-2.

Cell Culture and Reagents. CTLL-2 cells were acquired from the American Type Culture Collection (Rockville, MD). They were sustained in RPMI 1640 media (Sigma Chemical Company, St. Louis, MO) supplemented with 10% FBS, penicillin (50 IU/ml), streptomycin (50 mg/ml) (JRH Biosciences, Lenexa, KS) , gentamicin (50 mg/ml)

(Sigma), 2 mM L-glutamine (Gibco Labs, Grand Island, NY) and 20 mM HEPES (Sigma). This basal medium was supplemented with a final concentration of 10 mM α-methyl mannoside (Sigma) and 5% concanavalin A supernatant preparation from rat splenocyte culture (Rat T-Stim, Collaborative Research Inc., Bedford, MA) for the media used for CTLL-2 culture maintainance.

Evaluation of Leakage from Membranes. The levels of soluble IL-2 which could leak from the immobilized IL-2 membranes were determined as follows. Matrices prepared with radiolabeled IL-2 were incubated in basal media under culture conditions, with and without cells. For the case without cells, the supernatants were removed daily and replaced with media that had been incubating in parallel with the experiment. For the case with cells, 12 incubations were set up initially and four samples (supernatants) were removed and analyzed per day. The supernatants were counted in a gamma counter, and then mixed with 12% trichloro acetic acid (TCA) (1ml supernatant : 5ml TCA), set in ice overnight, and centrifuged at 1,000g for 30 minutes. The TCA supernatants were then disgarded and the pellets counted.

^{3}H-Thymidine Uptake Assay. CTLL-2 cells which have been incubated with ^{3}H-thymidine were eluted from culture by adding of a solution of 1.5mM ethylene-diamine-tetraacetic acid (EDTA) (Sigma) and 15% dimethylsulfoxide (DMSO) (Sigma) solution in PBS to the culture in a ratio of 0.5 ml of solution per ml of culture media (Van Oss *et al.*, 1983). This step was necessary to detach cells were bound to membranes. After addition of the EDTA-DMSO mixture, the cultures were agitated by repeated pipetting and then incubated at 37°C for 15 minutes. The complete contents of the culture wells were then transferred by sterile pipet into 96 well plate (Nunc immuno-plates, American Bio-Analytical, Natick, MA) and processed with a cell harvester so that cell lysates passed

through a glass fiber filter where the DNA was adsorbed. Samples were then analysed by liquid scintillation through a Packard 1600 CA liquid scintillation analyser (Sterling, VA).

Viable Cell Counting. Some of the immobilized IL-2 experiments described below were analyzed by using viable cell counts. Cells were eluted from the immobilized IL-2 culture experiments with EDTA-DMSO mixture as described above. The cell suspension was removed from the culture plate and the volume was measured by using a sterile volumetric pipet. An aliquot of the culture suspension was then mixed with an equal volume of trypan blue stain (0.4%) (Sigma) and viable cells were counted using a phase hemacytometer (Amrican Scientific Products, McGaw Park, Ill.).

CTLL-2 Sensitivity and Time Dependence on IL-2 Assays. The CTLL-2 time dependence experiment determined how long the cells could survive without the presence of IL-2 and was performed as follows. CTLL-2 cells were washed 3 times in basal media and resuspended in 1 ml of plain media in a 24 well culture plate (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ) at a density of 10,000 cells/well. IL-2 (1 ng in 0.1ml) was added to the wells at varying time intervals up to 72 hours after the initiation of culture. After 72 hours, 5 ng was added to all wells. ^{3}H-thymidine was pulsed into all wells 24 hours later. Samples were harvested for ^{3}H-thymidine uptake 24 hours after the pulse. All samples were run in duplicate.

The CTLL-2 IL-2 sensitivity analysis was run as follows. CTLL-2 cells were washed 3 times and resuspended in basal media at 100,000 cells/ml. Subsequently, 0.1 ml of this suspension was added to 1 ml of basal media that had been supplemented with variable amounts of IL-2 achieved by 3 fold sequential dilution of the media. After 72 hours, 5 ng of IL-2 in 0.1 ml of media was added to all wells and the analysis procedure followed that described immediately above. All samples were run in duplicate.

Immobilized IL-2 Assays. Immobilized IL-2 membranes and immobilized BSA membranes were washed 4 times in sterile PBS before use and placed in the bottom of 24 well cell culture plates with 1 ml of basal media added above. Autoclaved circles of supor-200 filter paper (pore size 0.2μm, Gelman Sciences, Ann Arbor, MI), 100 μm thick and 1.6 cm in diameter, were set atop some of the IL-2 membranes for leakage control experiments (Figure 1). The filter paper was measured to be 100 μm thick. Assuming a diffusion coefficient for IL-2 of 6×10^{-7} cm^2/s (the value for bovine serume albumin, a molecule 4 times larger than IL-2) the time required for the IL-2 to diffuse through the filter paper is given by t = (width of filter paper)^2/ diffusion coefficient. This is equal to 167 seconds in our system. Soluble IL-2 (1 ng/ml) was added to some of the culture wells with immobilized BSA membranes to show that the cells will proliferate in response to soluble IL-2 and that there is no toxicity from the membranes. These are the positive controls for the experiment (Fig. 1). Negative controls were run where the cells were incubated with the immobilized BSA membranes in the absence of IL-2 (Fig. 1). After loading the membranes and media, the culture plates were incubated at 37°C for 2 hours to equilibrate, and then 10,000 washed CTLL-2 cells in 0.1 ml of basal media were added to each well. Thirty-six hours later soluble IL-2 (5 ng in 0.1 ml) was added to each well. For the immobilized IL-2 assays which used ^{3}H-thymidine uptake as indicator of cell viability, ^{3}H-thymidine was pulsed into each well 24 hours after the addition of IL-2 to all of the wells. Cells were harvested for analysis 24 hours later. For the experiments which relied upon cell counting as an indicator of viability, no ^{3}H-thymidine was added. Cells were observed during the progress of the experiment by phase contrast microscopy using an Olympus CK2 inverted microscope (Olympus Optical Company, Tokyo, Japan).

2.3. Results

Characterization of Cells. The indicator cell line used in this study, CTLL-2, has been shown to proliferate in response to IL-2 and die in its absence (Gillis *et al.*, 1977). Because of considerations from our assay system, we established the sensitivity of CTLL-2 cells to soluble IL-2 and the amount of time the cells can be incubated without any IL-2 and still retain their proliferative potential. These experiments were performed to determine what levels of soluble IL-2 were permissable before any significant effect could be seen in our system.

Experiments to determine the length of time CTLL-2 cells can be incubated in media without any IL-2 and maintain their ability to incorportate thymidine were performed. Cells were washed and incubated in basal media for varying lengths of time, then rescued by the addition of soluble IL-2 (15 U to each ml of culture). ^{3}H-thymidine was added to the culture and the ability of the cells to incorporate the radiolabeled nucleotide is plotted as a function of time in IL-2 free media (Figure 2). As shown, an incubation for 32 hours in IL-2 free media results in the loss of proliferative potential by the cells as assessed by ^{3}H-thymidine uptake.

To determine the sensitivity of CTLL-2 cells to soluble IL-2 for maintaining viability, cells were washed and seeded in varying amounts of IL-2 and incubated for 72 hours. Soluble IL-2 was then added to all wells, followed by the addition of ^{3}H-thymidine and subsequent uptake analysis. As shown in Figure 3, the CTLL-2 cells require a minimum initial seeding concentration of .02 U/ml of soluble IL-2 to remain viable over the course of the assay.

Characterizarion of the Immobilized IL-2 Matrix. Non-porous immobilized IL-2 membranes were prepared as described in the Materials and Methods section. By varying the amount of radiolabeled IL-2 loaded in the coupling reaction, it was possible to prepare membranes from 50 ng to less than 1 ng of immobilied IL-2 per membrane, as measured by the presence of radiolabel. The distribution of IL-2 on the membranes was

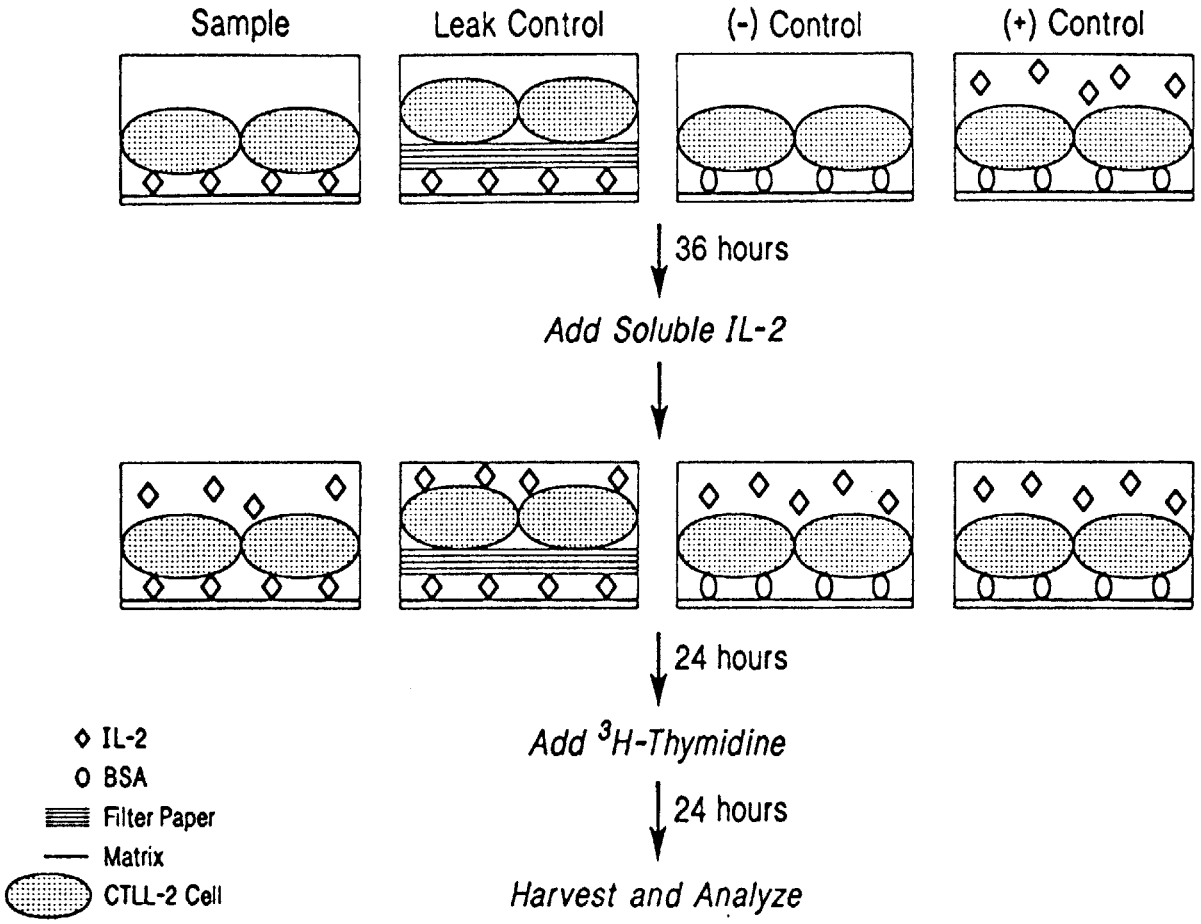

Figure 2.1. A schematic of the experimental procedures to determine the ability of the immobilized IL-2 membranes to preserve proliferative potential in CTLL-2 cells. The design of the experiments included 3 controls. The experimental samples had CTLL-2 cells incubating on top of the immobilized IL-2 matrix. Cells were incubated on top of membranes made with immobilized BSA protein, with and without added soluble IL-2, to serve as positive and negative controls. A control to monitor leakage from the membranes was also constructed by placing a piece of microporous filter paper, between the immobilized IL-2 membrane and the CTLL-2 cell layer.

Table 2.1. Determination of leaked protein activity*

Trials	Measured amount of IL-2 present (ng)	Calculated amount of leakage (ng)	Theoretical activity of leaked protein (Units)	Measured activity of leaked protein (Units)	% Activity
1	38.25	0.287	0.861	0.305	35
2	30.86	0.231	0.693	0.230	33
3	31.03	0.233	0.699	0.325	46
4	34.87	0.262	0.786	0.375	48
5	35.64	0.267	0.801	0.345	43
Average ± S.D.					41 ± 6.67

*Five immobilized IL-2 membranes were made with radiolabelled IL-2. These membranes were incubated in 1 ml of media for 3 days at 37°C. The supernatants were evaluated for IL-2 activity by CTLL-2 bioassay. Predicted activities were derived from the data shown in Figure 4.

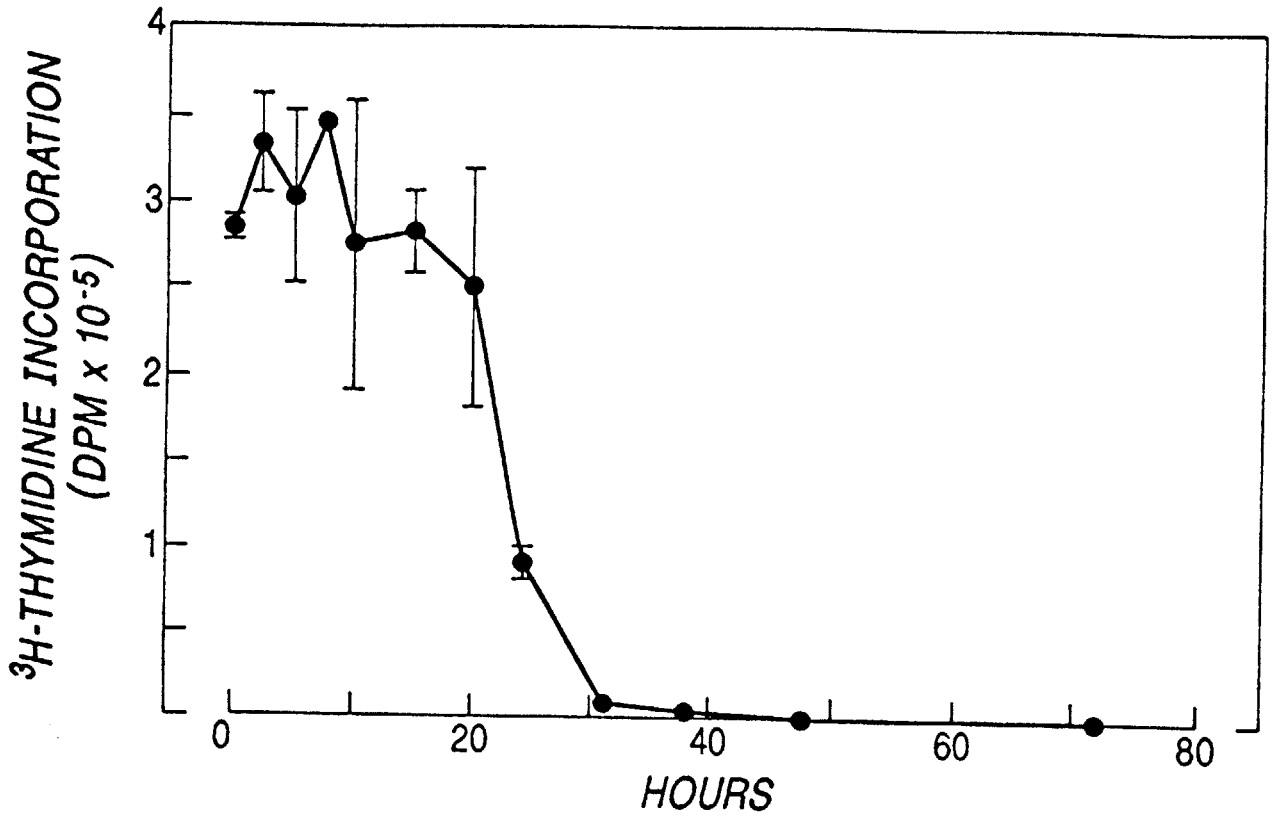

Figure 2.2 Time course of the ability of CTLL-2 cells to maintain proliferative activity in IL-2 starved culture. Parallel wells with 10,000 CTLL-2 cells/ml were seeded in basal media. At the times indicated on the bottom axis, 1 ng of IL-2 in 0.1ml of media was added to each well. After 72 hours 5 ng of IL-2 in 0.1 ml of media was added to all wells. Twenty four hours later, 0.5 mCi of ^{3}H-thymidine was added to all wells and cells were harvested after another 24 hours for thymidine incorporation. As shown, an incubation for 32 hours in IL-2 free media results in the loss of proliferative potential by the cells as assessed by ^{3}H-thymidine uptake.

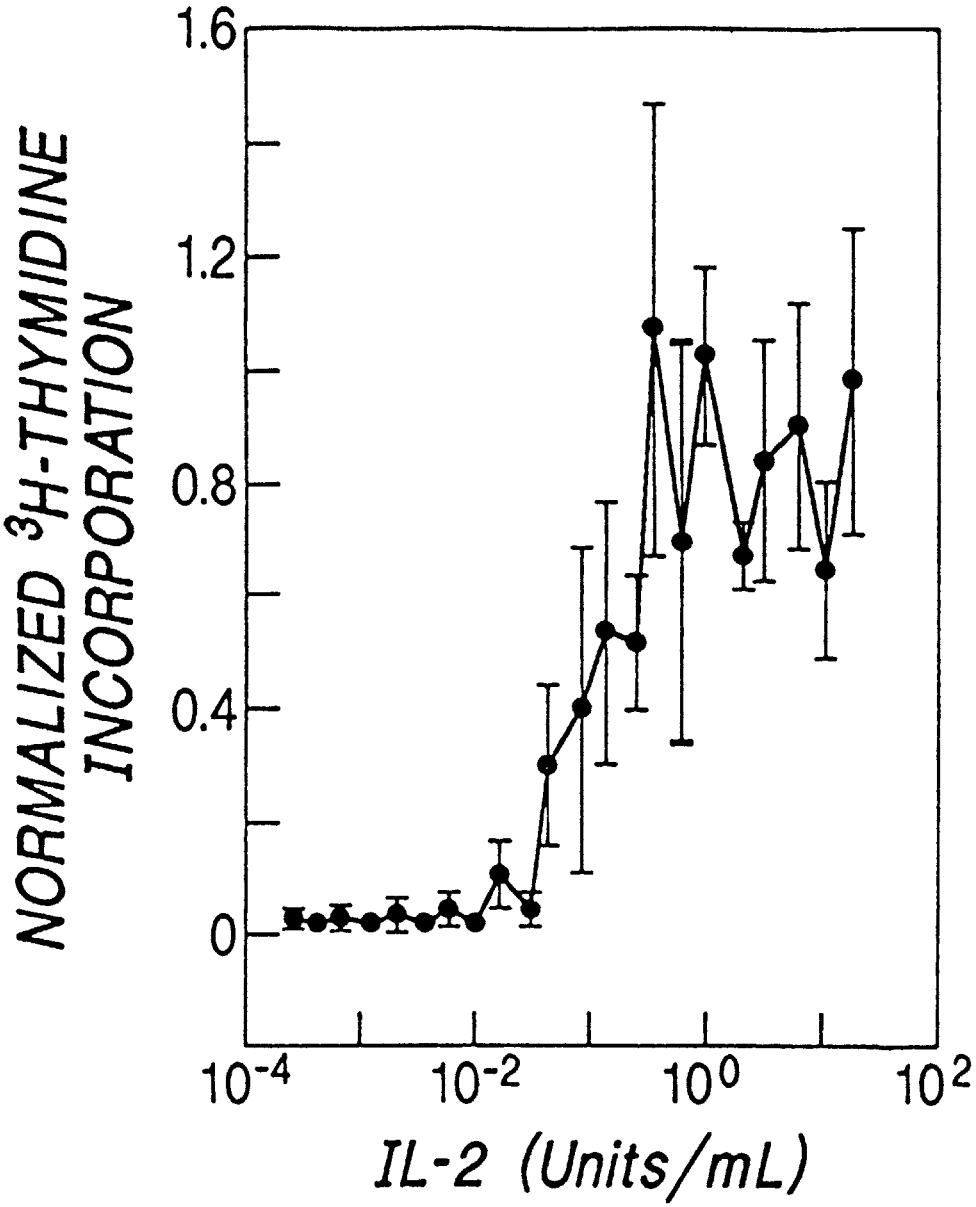

Figure 2.3. Soluble IL-2 requirement of CTLL-2 cells to maintain proliferative potential. Ten thousand CTLL-2 cells/well were incubated in 1 ml of media with IL-2 concentrations that were serially diluted from 20 U/ml down to under .0001 U/ml. 72 hours after seeding, 5 ng of IL-2 in 0.1 ml of media was added to all wells. 24 hours later, 0.5 mCi of ^{3}H-thymidine was added to all wells and samples were harvested for analysis after another 24 hours. The amount of thymidine incorporation was normalized by the amount incorporated at 1.1 U/ml initial concentration, roughly the maximum point for all trials. Each point shows the mean and standard deviation for 2 to 6 replicate trials. As shown, CTLL-2 cells require a minimum initial seeding concentration of .02 U/ml of soluble IL-2 to remain viable over the course of the assay.

homogeneous according to autoradiographic exposures of membranes prepared with ^{125}I IL-2 (data not shown).

A continual concern was that some appreciable amount of IL-2 was adsorbed (not covalently bound to the solid matrix) and capable of desorbing from the membranes during the assay procedure. To check for this possibility, experiments were performed with membranes prepared from radiolabeled IL-2. The membranes were incubated under assay conditions but without the addition of cells, and the supernatants were collected, TCA precipitated, and checked for radiolabeled protein. These results are shown in Figure 4 and are plotted as % of immobilized protein present at the start of the experiment that was lost during the procedure. Figure 4 also shows a similar experiment where the membranes were incubated with 10,000 CTLL-2 cells to observe whether the desorption phenomenon is enhanced with cells present. The data is given as the TCA precipitable activity present in the supernatants. The figure shows that there are no significant differences in desorption of radiolabeled protein between the incubations with and without cells. The leakage rate in either case is approximately 0.25% of immobilized protein lost / day.

In addition to quantitating the amount of IL-2 leaked, the activity of the protein which leaked off from the immobilized IL-2 matrix was determined. Radiolabeled immobilized IL-2 membranes were prepared and the amount of IL-2 present on the membranes was determined. Each membrane was incubated in 1 ml of basal media at 37 °C for 3 days, and then the supernatants were collected. From the relationship shown above in Figure 4, it was calculated that 0.75% of the immobilized IL-2 should have leaked off from the membranes in this time. If we assume that all of the leaked IL-2 was active, we arrive at the predicted value for the IL-2 activity of these supernatants. These supernatants were then assayed by CTLL-2 cells to determine the actual measured activity of the samples. As shown in table 1, the measured activity of leaked IL-2 is about 40% of the predicted activity if all of the leaked protein were active.

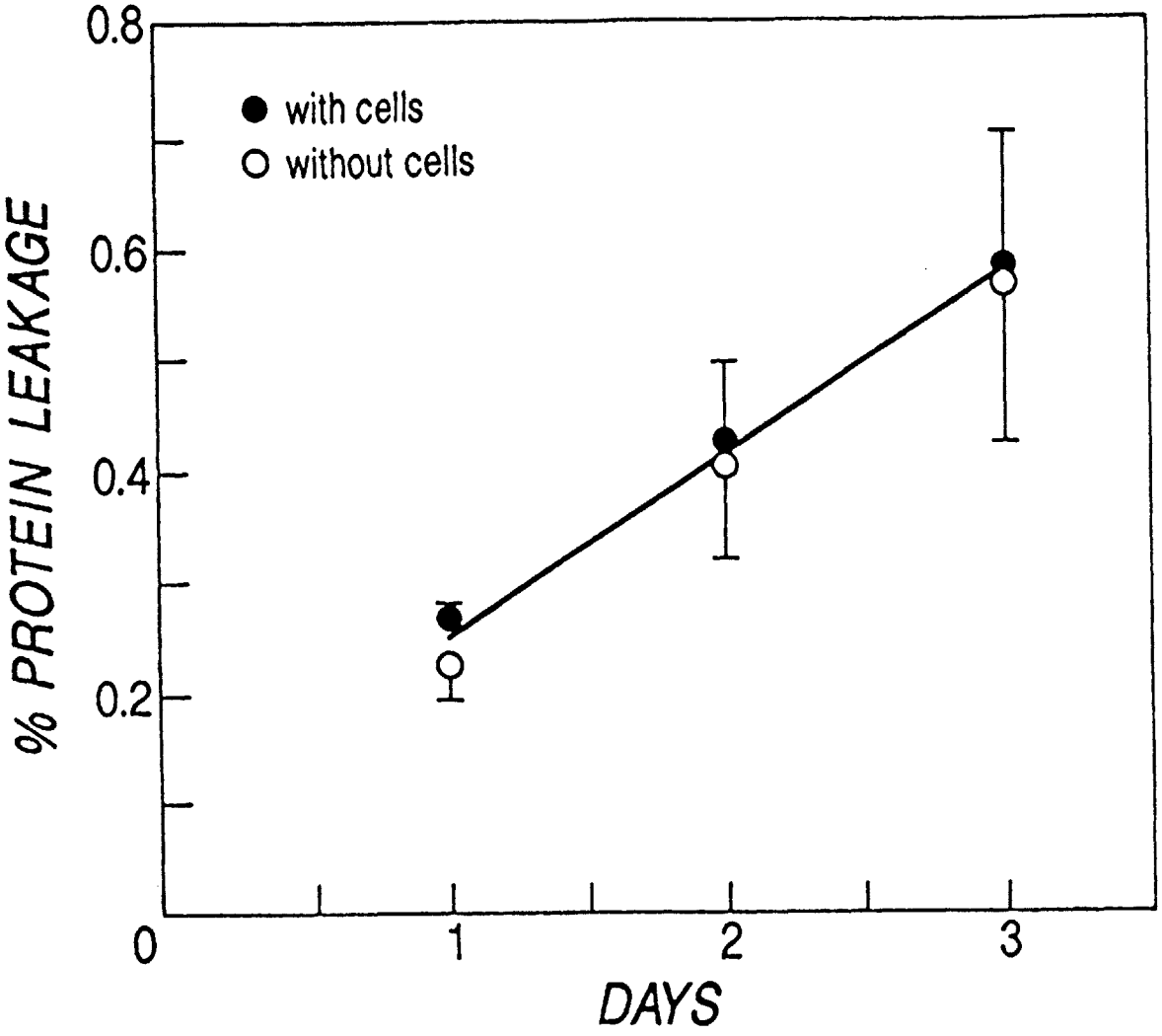

Figure 2.4. Leakage of TCA precipitable radiolabelled protein from immobilized IL-2 membranes. The amount of TCA precipitable protein which desorbed from the immobilized IL-2 matrices during 2 studies. Open circles refer to incubation where no cells were present and data represents cumulative loss over the time period indicated. Each point shows the mean and standard deviation of 5 replicate trials. Closed circles were samples that were incubated with 10,000 CTLL-2 cells / sample. Data represents 4 replicates per time point. The figure shows that there are no significant differences in desorption of radiolabeled protein between the incubations with and without cells. The leakage rate in either case is approximately 0.25% of immobilized protein lost / day.

Immobilized IL-2 Assays. The immobilized IL-2 matrices were evaluated for their effects on CTLL-2 cells. The design of the experiments included 3 controls and is shown schematically in Figure 1. The experimental samples had CTLL-2 cells incubating on top of the immobilized IL-2 matrix. Cells were incubated on top of membranes made with immobilized BSA protein, with and without added soluble IL-2, to serve as positive and negative controls. A control to monitor leakage from the membranes was also constructed by placing a piece of microporous filter paper, between the immobilized IL-2 membrane and the CTLL-2 cell layer. By placing very small volumes of IL-2 on one side of the filter paper and a cell layer on the other, it has been demonstrated that IL-2 can efficiently diffuse through the filter paper. Using .03 U of IL-2, the cell layer incorporated three times the tritiated thymdine as a negative control (data not shown). The amount of time required for soluble IL-2 to diffuse across the filter paper has been calculated to be less than 10 minutes.

To determine if the immobilized IL-2 matrix could support proliferation of the CTLL-2 cells, samples and controls were incubated for 48 hours, pulsed with ^{3}H-thymidine, and harvested 24 hours later. The results from these experiments showed that cells incubated directly on top of the immobilized IL-2 matrix incorporated only slightly more ^{3}H-thymidine than did the negative controls (cells without any IL-2 incubating on an immobilized BSA membrane) (data not shown). This was an extremely puzzling result since, by phase contrast microscopy, those cells incubating on the immobilized IL-2 membrane looked to be large and fully rounded, with smooth cytoplasms, and distinct cell membranes- characteristic of healthy cells- whereas the negative controls were much smaller, flat, and granular with irregular cell membranes - characteristic of dead cells. To show that the immobilized IL-2 matrix had, in fact, maintained the viability of the incubating cells, soluble IL-2 was added to all wells after 36 hours and ^{3}H-thymidine was pulsed after 60 hours. Cells were harvested for analysis 84 hours after the start of the culture experiment. As can be seen in Figure 5, the immobilized IL-2 membrane preserved

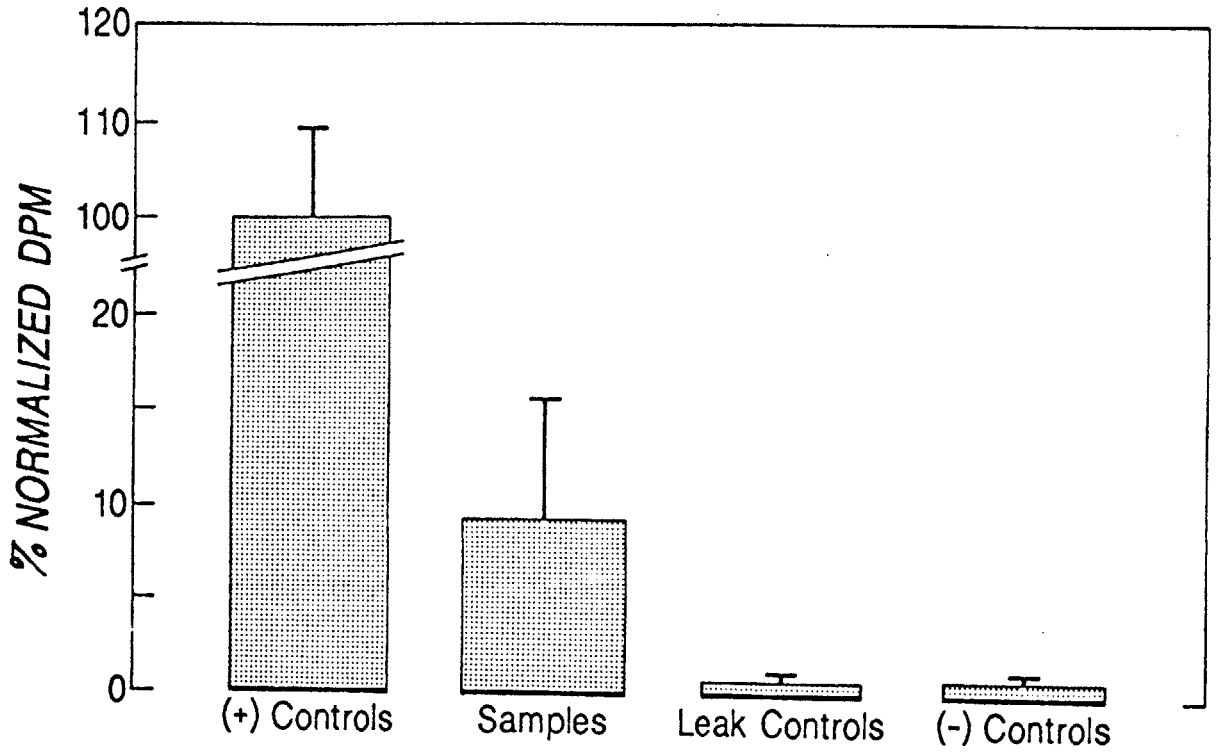

Figure 2.5. To show that the immobilized IL-2 matrix had maintained the viability of the incubating cells, samples and controls were incubated with their matrices for 36 hours. Soluble IL-2 was then added to all wells and ^{3}H-thymidine was pulsed after 60 hours. Cells were harvested for analysis 84 hours after the start of the culture experiment. The experimental procedure is schematized in Figure 1. Data shown are the mean and standard deviation for the listed number of replicate trials. The data has been normalized to the positive controls. The actual number for a typical sample trial was around 50,000 dpm. As can be seen, the immobilized IL-2 membrane preserved the viability and proliferative potential of CTLL-2 cells whereas the immobilized albumin membrane did not. The leakage control was not significantly different from the negative control. Sample values are significantly different from the negative and leak control values ($p < 0.05$ by t-test)

the viability and proliferative potential of significantly more cells than did the immobilized albumin membrane. The leakage control was not significantly different from the negative control. Figure 5 shows the results of an experiment where the cells were assayed by ^{3}H-thymidine measurement and the results are presented as % normalized DPM. The samples incubatedwith the immobilized IL-2 membrane incorporate significantly more ^{3}H-thymidine than the leakage controls or the negative controls ($p < 0.05$ by t-test). In Figure 6, the ^{3}H-thymidine pulse was omitted and viable cell counts were performed instead. As is shown, there were significantly more viable cells from the immobilized IL-2 membrane incubation than with either the negative control or the leakage control ($p < 0.02$).

2.4. Discussion

The question as to whether a growth factor which is active in the soluble state is capable of mediating activity when it is immobilized to an insoluble matrix and contacted with sensitive cells was first investigated more than 2 decades ago (Cuatrecasas, 1969). In the present study, a system to explore this question using a novel immobilized IL-2 matrix and the IL-2 dependent cell line CTLL-2 was developed. The immobilized IL-2 membranes were shown to sustain the viability of CTLL-2 cells over a 36 hour incubation. These cells could then be stimulated to proliferate and incorporate ^{3}H-thymidine when treated with soluble IL-2. However, if soluble IL-2 was not added, the culture incorporated only negligible amounts of ^{3}H-thymidine even though the cells appeared to be healthy under microscopic examination. This implies that the immobilized IL-2 matrix does not induce the proliferation of CTLL-2 cells in the manner that soluble IL-2 does. Thus the immobilized IL-2 membrane appears to decouple the effects of proliferative stimulation and maintainance of viability which is caused by soluble IL-2. Our findings also suggest that internalization of the bound IL-2 - IL-2 receptor comples is not necessary for the mediation of at least some of the effects of IL-2.

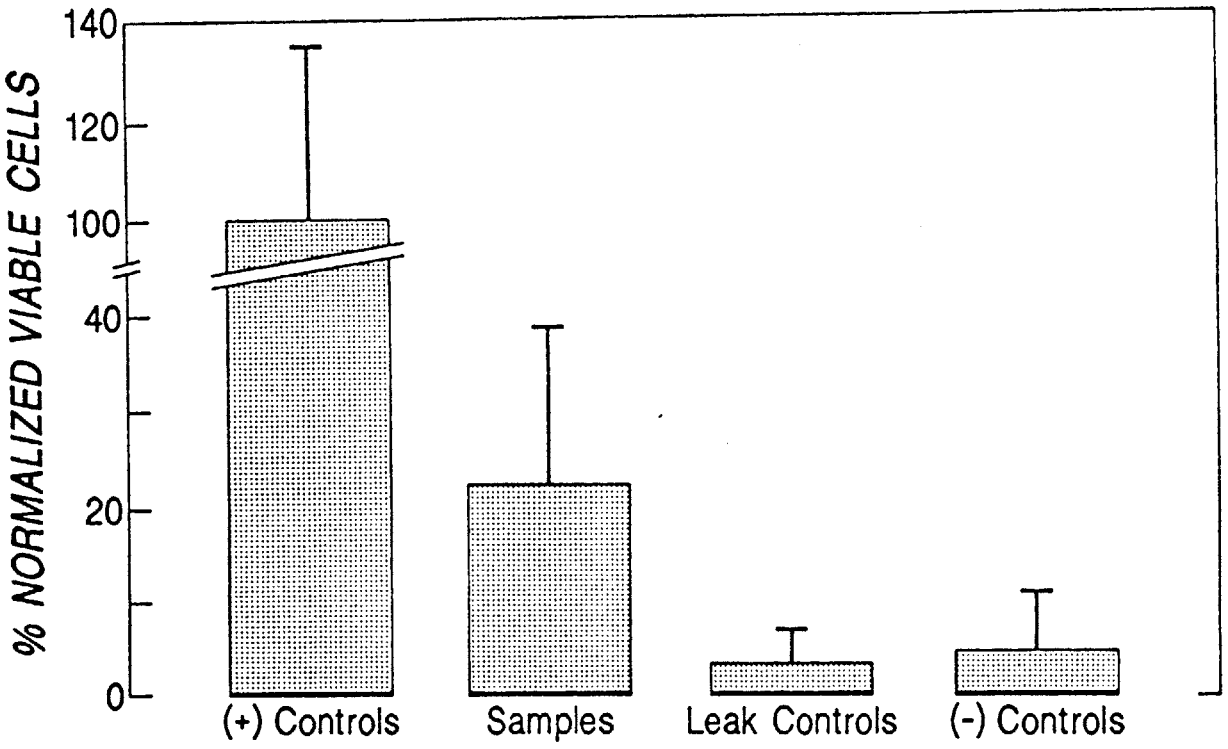

Figure 2.6. In this experiment, the ^{3}H-thymidine pulse was omitted and viable cell counts were performed instead to analyze the preservation of viability by the immobilized IL-2 membrane. As is shown, there were significantly more viable cells from the immobilized IL-2 membrane incubation than with either the negative control or the leakage control ($p < 0.02$).

Although it is not known how the immobilized IL-2 matrix mediates this effect, there are many possibilities. With exposure to soluble IL-2, the IL-2 sensitive cell can bind IL-2 to a specific receptor, the bound receptor-ligand complex can then migrate or diffuse along the cell surface to some site where the complex can be internalized. By immobilizing the IL-2, one ostensibly allows the receptor-ligand binding reaction while preventing lateral diffusion and internalization of the complex. It is plausible that by preventing either the free lateral diffusion or the internalization of the receptor complex, one inhibits some features of the signalling mechanism IL-2 generates in its soluble form. It is also a possibility that the binding of receptor and ligand is a sufficient and complete signal but, due to the low surface density of IL-2 and the loss of activity of the protein as it undergoes the coupling reaction, there may not be enough receptors occupied by active IL-2 molecules to generate detectable proliferation. Another alternative is that cells require soluble IL-2 for all effects and achieve this in our system by enzymatically cleaving IL-2 from the membrane or extracting bound protein by other, non-spontaneous means. To rule out the possibility that spontaneous leakage of IL-2 from our immobilized IL-2 matrix could mediate any of the observed biological effects, we have performed a series of experiments to carefully characterize our system and to measure the leakage rate.

We found that our membrane system leaks 0.25% of the immobilized protein per day and that only 40% of this leaked protein is active. In addition, the indicator cell line, CTLL-2 was characterized in terms of how long these cells can survive without IL-2 and at what critical concentration of soluble IL-2 the cells will remain viable. We found that after 32 hours without soluble IL-2, a CTLL-2 culture loses all viability and that a culture requires a concentration of at least 0.02 U/ml to remain alive. Using these criteria, immobilized IL-2 membranes that contain 4.8 ng or less of the protein could be used without worry that the effect observed for a 36 hour incubation was due to leakage of IL-2. A 5 ng membrane was estimated to have a density of about 500 IL-2 molecules/μm^2 or

77,000 molecules under the projected area of a cell (diameter ~ 15 μm). Considering that there are from 10-30,000 high affinity IL-2 receptors per CTLL-2 cell (Robb *et al.*, 1981), enough IL-2 is present to saturate all of the receptors with 200% excess if all of the immobilized IL-2 is capable of binding to receptors. This loading also respresents about 1% of a monolayer on our non-porous support.

As a second check that the immobilized IL-2 membranes does not leak enough IL-2 to cause the observed biological effect, we have included a leak control in all of the immobilized IL-2 studies. This control includes a piece of filter paper of 100 μm thickness and 0.22 μm pore size that is placed between the cell layer and the immobilized lymphokine matrix. This set up allows the cell layer to respond to any IL-2 which has leaked off the membranes without coming into contact with the immobilized molecules. The calculated amount of time it would take for an IL-2 molecule that was soluble to diffuse from the membrane surface to the cell layer is less than 10 minutes and the system is pre-incubated for 2 hours before cells are loaded. In all of the included trials these controls were negative for the presence of soluble IL-2. In summary, leakage characterization studies showed clearly that the observed effect of IL-2 membranes on CTLL-2 cells to preserve the viability of these cells was a real biological phenomenon.

There are many elements of interest in our system which should be rewarding of future study. Although the IL-2 molecules covalently bound to the matrix ostensibly cannot be internalized by the cells, we would like to show this directly by performing internalization studies with radio-labeled IL-2. It is possible that the IL-2 receptor complexes expressed by the CTLL-2 cells are being internalized in some fashion, and it would be interesting to follow the intracellular and surface levels of the receptor complexes. We would also like to investigate the properties of IL-2 dependent intracellular signalling, and to determine whether the density or clustering of IL-2 receptor complexes is an important parameter with regard to the intracellular signal transduction. Some aspects of the effects of the immobilized lymphokine matrix are not easily analyzed at this time because of the

limitations of materials. IL-2 is a difficult protein to immobilize because of its tendency to self agglomerate and adsorb to surfaces when no carrier protein is present (Kaplan, 1991). These limitations required that a matrix of relatively sparse coverage (1% of a monolayer) be prepared for the studies to avoid complications from the spontaneous desorption of IL-2 during experimental trials. Efforts are underway to resolve these issues by using new techniques of immobilization that would allow greater loadings of IL-2 per unit surface area while still avoiding leakage. With these tools, it should be possible to quantitatively explore other aspects of the interaction between IL-2 dependent cells and the immobilized IL-2 matrix.

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CHAPTER 3

CHARACTERIZATION OF IMMOBILIZED IL-2 TREATED CELLS

3.1. Introduction

In the previous chapter it was established that an immobilized IL-2 membrane is capable of preserving the viability of CTLL-2 cells in culture without apparently causing cell growth. These cells were shown to die completely after 30 hours when no IL-2 was present, but they remained viable on the immobilized IL-2 membrane for up to 36 hours. The CTLL-2 cells on the immobilized IL-2 membrane were also found to be a non-proliferating population; these cultures incorporated only very limited amounts of ^{3}H-thymdine in spite of the very large numbers of viable cells present. This activity of immobilized IL-2, to support viability without causing growth, is novel and and thus far, unique. In this chapter, experimental studies will be presented which better define the state of the CTLL-2 cells incubated with the immobilized IL-2 membrane.

It is necessary to better understand the effects of the immobilized IL-2 membrane on the cells for a two reasons. The first is that the use of immobilized IL-2 in culture apparently is capable of mediating at least one novel effect on sensitive cells. It is interesting to explore if other novelties might also be present that would go undetected without searching for them. The second reason is that the immobilized IL-2 membrane has already been shown to have some useful properties in terms of providing an affinity matrix for cells that express the IL-2 receptor and for the biological activity mentioned above. However, it would not be appropriate at this time to use such a system for cell separations or long term cell culture or as a substitute for IL-2 if the immobilized IL-2 membrane altered cell properties in unknown or unexpected manners. To determine how best to utilize this system, it must be better understood what the system does to

IL-2 sensitive cells.

The studies chosen in this section each address an aspect of cellular behavior that is both interesting and useful for understanding the effects of the immobilized IL-2 membrane. We have performed cell cycle analysis of CTLL-2 cells incubated on the immobilized IL-2 membrane because the membrane has been shown to arrest cellular proliferation while maintaining viability. These procedures will answer the question of whether the immobilized IL-2 membrane causes cells to be blocked in one section of their cell cycle or if the membrane prevents cell cycle progression. It is also very interesting to know how other IL-2 mediated functions are affected by the immobilized IL-2 membrane. For the cell line we are using, there is no obvious effector function that can be assayed, so it was appropriate to examine the expression levels of an IL-2 stimulated cell protein. The protein examined was the IL-2 receptor α chain which is quickly upregulated by soluble IL-2 and which also disappears quickly from cells when no IL-2 is present. The major use of CTLL-2 cells is that they serve as a indicator cell line for the presence of IL-2. Their utility in bioassays is due to their ability to proliferate at a rate proportional to the amount of IL-2 present. To determine if incubation with the immobilized IL-2 membrane might alter cellular properties, we assayed the growth response of the CTLL-2 cells to soluble IL-2 for control populations and for cells incubated with the immobilized IL-2 membrane. Finally, a pressing question about the ability of the immobilized IL-2 membrane to preserve viability without apparently supporting growth was whether IL-2 has these distinct activities, preserving viability and causing growth, that the membrane was somehow able to divorce, or whether the preservation of viability was a growth related phenomenon and that the incubating population was simple growing very slowly. To answer this question, CTLL-2 cells were treated with Mitomycin C to block their proliferation and then assayed for viability with and without IL-2.

3.2. Materials and Methods

Preparation of Membranes. Immobilized IL-2 membranes were prepared starting from MatTek T2 membranes (MatTek Corp., Ashland, MA). These are nonporous, polystyrene materials which have been gas plasma treated to provide modified surfaces. The surfaces include primary amino groups which were used for the immobilization procedure. The membranes were cut into 1.5 cm diameter circles and washed through an ethanol gradient ranging from 95% to 0% ethanol in water. The circles were then treated with 2% glutaraldehyde for 30 minutes, washed twice in sterile phosphate buffered saline (PBS), pH 7.4, and then treated overnight with 100 ng of human recombinant IL-2 (a gift from DuPont, Wilmington, DE) suspended in 2 ml of PBS. Some membrane preparations used a radiolabeled IL-2 tracer (New England Nuclear, Boston, MA) in the coupling reaction for quantitation of the amount of IL-2 bound. It has been determined through studies using radiolabeled IL-2 that membranes treated with 100 ng in 2 ml of PBS yield final amounts of 4.8 ng or less of immobilized IL-2. After coupling, the membranes were passed through 3 washes in sterile PBS and 3 washes of sterile PBS + 10% (v/v) fetal bovine serum (FBS). Each wash was for a 24 hour period. The membranes were kept at 4 °C for the first 20 hours of the wash but were then moved to a 37 °C incubator for the final four hours. Subsequently, membranes were stored in sterile PBS + 10% FBS at 4°C until use. Membranes could be stored for a month before use with no discernable loss of activity.

Cell Culture and Reagents. CTLL-2 cells were acquired from the American Type Culture Collection (Rockville, MD). They were sustained in RPMI 1640 media (Sigma Chemical Company, St. Louis, MO) supplemented with 10% FBS, penicillin (50 IU/ml), streptomycin (50 mg/ml) (JRH Biosciences, Lenexa, KS) , gentamicin (50 mg/ml) (Sigma), 2 mM L-glutamine (Gibco Labs, Grand Island, NY) and 20 mM HEPES (Sigma). This basal medium was supplemented with a final concentration of 10 mM α-

methyl mannoside (Sigma) and 5% concanavalin A supernatant preparation from rat splenocyte culture (Rat T-Stim, Collaborative Research Inc., Bedford, MA) for the media used for CTLL-2 culture maintainance. P815 cells were acquired from the ATCC and were maintained in Dulbecco's modified eagles' media with 10% fetal bovine serum and incubated at 37°C with 10% CO_2.

Mitomycin C treatment of CTLL-2 cells. Mitomycin C (Sigma) was suspended in plain RPMI 1640 media at a concentration of 1 mg/ml and distributed into 100 µl aliquots in microvials that were wrapped in foil and stored at -20 C. When needed, a vial would be thawed and pipetted into 5 ml of basal media that would be filter sterilized by loading the volume into a 7 ml syringe and injected through a Millex-GV 0.22 µm filter unit (Millipore, Bedford, MA) into a sterile 12 ml centrifuge tube. CTLL-2 cells in their maintainance media would be washed three times in basal media and then resuspended in 2.5 ml of the Mitomycin C media at a concentration of about 40,000 cells/ml in 25 cm^2 culture flasks (Falcon, Franklin Lakes, N.J.) and incubated at 37°C. After 2 hours, the cells were removed and washed twice in basal media before being resuspended under the appropriate conditions. Control cultures were run in parallel to the Mitomycin C treated cells where the controls were incubated with plain basal media for 2 hours.

Viable Cell Counting. For the viable cell count assays, Mitomycin C treated CTLL-2 cells were prepared as described above and then resuspended in basal media that was either supplemented with soluble IL-2 (1ng/ml) or contained no IL-2. A 100 µl sample of the indicated cell culture was removed at the indicated times and mixed with an equal volume of trypan blue stain (0.4%) (Sigma). Viable cells were counted using a phase hemacytometer (American Scientific Products, McGaw Park, Ill.) and the total number of viable cells was calculated based on the original volume of the cell culture and the dilution of the sample due to trypan blue.

Mitomycin C ^{3}H-Thymidine Uptake Assay. Mitomycin C cultures and controls were prepared as described above and resuspended in media containing either no IL-2 or soluble IL-2 (1ng/ml). These samples were incubated at 37°C for 36 hours and then pulsed with ^{3}H-thymidine at 0.5 μCi/ml for 24 hours. The complete contents of the culture flasks were then transferred by sterile pipet into 96 well plates (Nunc immuno-plates, American Bio-Analytical, Natick, MA) and processed with a cell harvester so that cell lysates passed through a glass fiber filter where the DNA was adsorbed. Samples were then analysed by liquid scintillation through a Packard 1600 CA liquid scintillation analyser (Sterling, VA).

Immobilized IL-2 Incubations. Immobilized IL-2 membranes and were washed 4 times in sterile PBS before use and placed in the bottom of 24 well cell culture plates with 1 ml of basal media added above. After loading the membranes and media, the culture plates were incubated at 37°C for 2 hours to equilibrate, and then 40,000 washed CTLL-2 cells in 0.1 ml of basal media were added to each well. After the indicated incubation times, cells were eluted from the immobilized IL-2 membrane by adding 0.5 ml of 15% DMSO and 1.5 mM EDTA in basal media per ml of media in each well and incubating for 15 minutes. These reagents aid in dissociating adherent cells from the immobilized IL-2 membrane. The culture media was then agitated by repeatedly filling and disbursing the culture with a pipet to rinse off adherent cells from the membrane before finally transfering the culture to a sterile centrifuge tube and washing the cells once in basal media. Viable cells were collected after the first wash by suspending the cells in 10 ml of basal media and centrifuging the suspension at 10 x g (250 rpm at 20 cm radius) for 10 minutes, collecting the pellet and discarding the supernatant. Trypan blue staining confirmed that the resulting cells were viable. These cells would then be used for the assays discussed below or for transfer to new incubation conditions in continuing experiments. Controls cell populations would be plated in parallel to the immobilized IL-2 samples. The control sample media

might contain soluble IL-2 at 1 ng/ml ((+) controls) or no IL-2 ((-) controls). Control samples were plated in the same 24 well culture plates as the immobilized IL-2 samples and were eluted in an identical fashion.

Propidium Iodide Staining for Cell Cycle Analysis. The propidium iodide staining of Mitomycin C treated CTLL-2 cells, control cell incubations and immobilized IL-2 incubations used the method described by Cissman (1). Briefly, cells were harvested and washed as described above and check for viability. They were then washed twice in PBS and each sample was resuspended in PBS + 1% serum in a sterile 15 ml centrifuge tube. While vortexing, 10 ml of 95% ethanol was added, and the tubes were stored at 4°C for at least 12 hours and up to 4 days (cells may remain in fixative for up to a week). The fixed cells were then spun down and washed twice in PBS + 1% serum before being resuspended in 0.9 ml of PBS + 1% serum. 0.1 ml of 10x propidium iodide (Sigma) solution (50 μg/ml of propidium iodide in .04M sodium citrate buffer, pH 7.0, stored wrapped in foil) was then added to each tube along with 20 μl of RNAase suspension (RNAase (Sigma) suspended in PBS at 10 mg/ml). The samples were then kept at room temperature for 1 hour before analysis on a FACS. Analyses of the cell cycle distribution of cells presented in column graphs was performed by obtaining an elarged hard copy of the histogram output from the FACS machine and then cutting out the profile and the relevant peaks with scissors. The peaks were then weighed on a Mettler balance and compared to the total weight of all of the peaks to arrive at the relative weighing.

Anti-IL-2R α Chain Staining. CTLL-2 cell samples were harvested from their respective incubations as described above and washed twice in PBS + 3% serum. The cells were then resuspended in PBS + 2.5% normal rat serum and incubated at 4°C for 15 minutes. The samples were then again washed in PBS + 3% serum and concentrated into a 50 μl pellet. To this pellet was added 50 μl of fluorescein labeled rat-anti-mouse anti-IL-2R α chain

monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) and the samples were incubated 15 minutes at 4°C. Samples were then washed twice in PBS + 3% serum and placed on ice until they were analyzed, as soon as was possible, in a FACS. Statistical anlyses of the histograms was performed by the Consort software package of the FACS. Staining controls followed the same procedure except for using a rhodamine labeled rat-anti-mouse IgG monoclonal antibody for staining or by using P815 cells instead of CTLL-2 (2-4).

IL-2 Titrations of CTLL-2 Cells Incubated with Immobilized IL-2. CTLL-2 cells were incubated with immobilized IL-2 and harvested as described above. The cells were then concentrated and resuspended in basal media to a final concentration of 5-10,000 cells / 100 µl. 100 ml of the cell suspension was then added to the wells of a 96 well plate that already contained 100 ml of media plus titrated amounts of soluble IL-2. The plates were incubated for 18 hours at which time 0.5 µCi of 3H-thymidine (New England Nuclear, Boston, MA) was added to all wells. Twenty four hours later, the plates were processed and analyzed in a cell harvester and by liquid scintillation counting as described above. All samples were run in duplicate and all experiments were repeated twice.

3.3. Results

Mitomycin C Treatment of CTLL-2 Cells. The results from our immobilized IL-2 assays have shown that the immobilized IL-2 matrix can maintain the viability of CTLL-2 cells even though the cell population is apparently non-proliferative. To better determine whether CTLL-2 cells can remain viable in a nonproliferative state, the cells were treated with the reagent Mitomycin C. This drug cross-links DNA and thus prevents the separation of the daughter strands during mitosis and the efficient replication of DNA

during the synthesis phase of the cell cycle. Thus cells treated with Mitomycin C are locked into the S or M phases of the cell cycle and cannot undergo cellular division.

To establish that the Mitomycin C does not kill CTLL-2 cells, we treated a sample population with 20 μg/ml for 2 hours and then washed the cells and resuspended them in media with and without IL-2 (1 ng/ml). Viable cell counts, obtained by trypan blue staining and a haemocytometer, were then used to establish the viability of the populations. A control was run with CTLL-2 cells that were treated for 2 hours with plain media and then also washed and resuspended in media with and without IL-2. The results are shown in Figure 3.1. As can be seen, the cells that were pretreated with Mitomycin C maintained a fairly constant level of viable cells throughout the course of the procedure. The control group initially declined in cell number, but then resumed their proliferative behavior. Therefore, treatment of CTLL-2 cells with Mitomycin C for 2 hours results in a viable cell population for at least 56 hours after treatment when the cells are cultured with IL-2 and is not demonstrably toxic to the cells. For both the Mitomycin C treated and the control cell populations, incubation in IL-2 free media resulted in undetectable numbers of viable cells and thus are not included in the figure.

Although the results shown in Figure 3.1 suggest that Mitomycin C treatment of CTLL-2 cells has resulted in a viable non-proliferative state for the cells, these results could also be interpreted as a dynamic equilibrium where cells are proliferating and dying at roughly the same rate. To rule out this possibility, we analyzed the cell populations for their ability to incorporate ^{3}H-thymidine. If the cells are actively replicating their DNA, their ^{3}H-thymidine signal should be significant. Conversely, if the cells are arrested in their cell cycles, the amount of ^{3}H-thymidine incorporation should be negligible. As shown in Figure 3.2, cells that were pretreated in Mitomycin C (20 μg/ml for 2 hours), kept in culture for 36 hours, and then pulsed with 0.5 μCi /ml of ^{3}H-thymidine for 24 hours incorporated only a minimal amount of ^{3}H-thymidine when incubated with 1 ng/ml of IL-2, whereas the untreated control population generated a significant signal in the IL-2

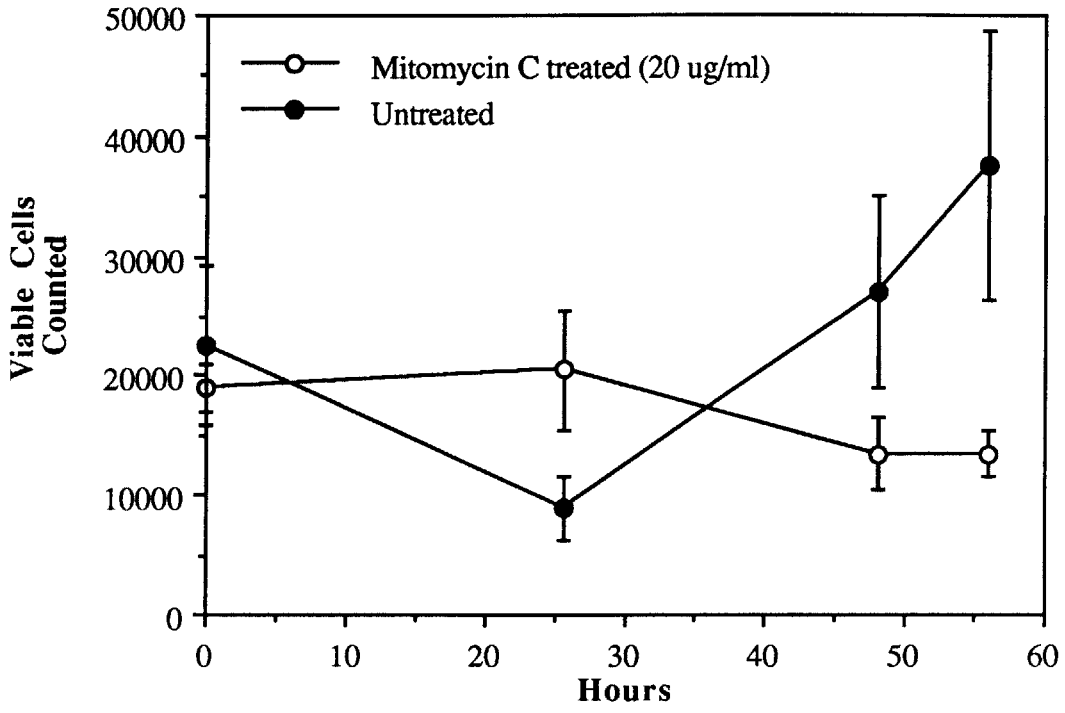


Figure 3.1 Viable Cell Counts of Mitomycin C treated CTLL-2 Cells.
20,000 CTLL-2 cells were incubated in 20 μg/ml of Mitomycin C or in plain media for 2 hours. The cells were then washed and resuspended in media with IL-2 (1 ng/ml). At the indicated times, an aliquot of the cultures was sampled and the number of viable cells was determined by staining the cells with trypan blue and counting in a haemocytometer. Samples were run in duplicate and the given points represent the means and standard deviation of the data. A parallel set of experiments was run where cells were resuspended and incubated in IL-2 free media. There were no viable cells apparent at the 25 hour time point in these trials.

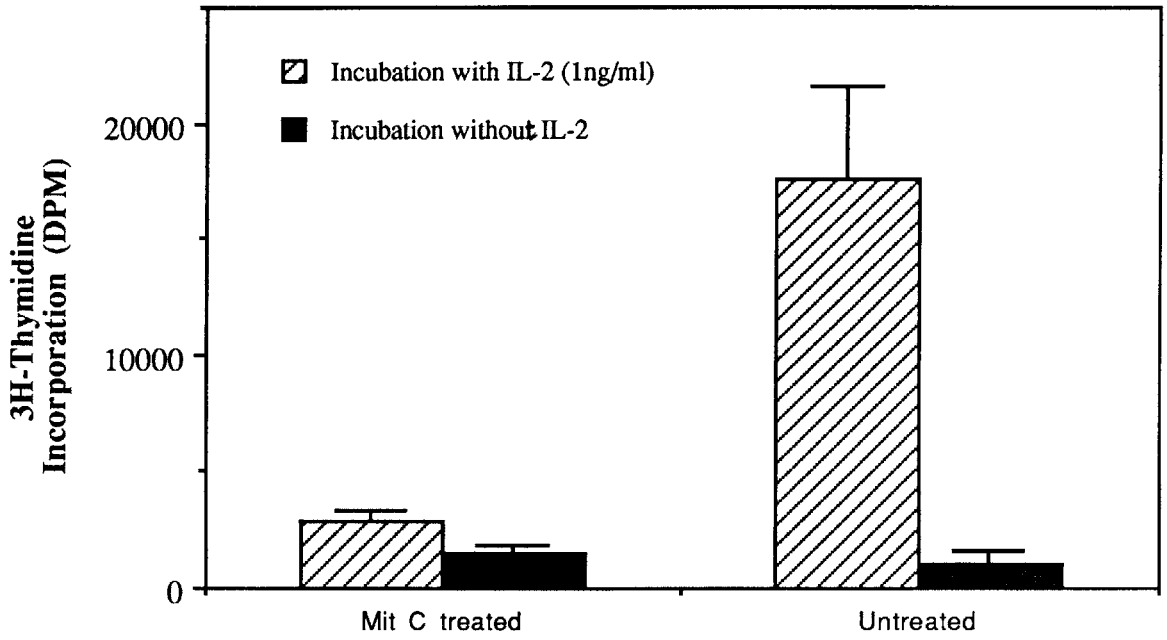
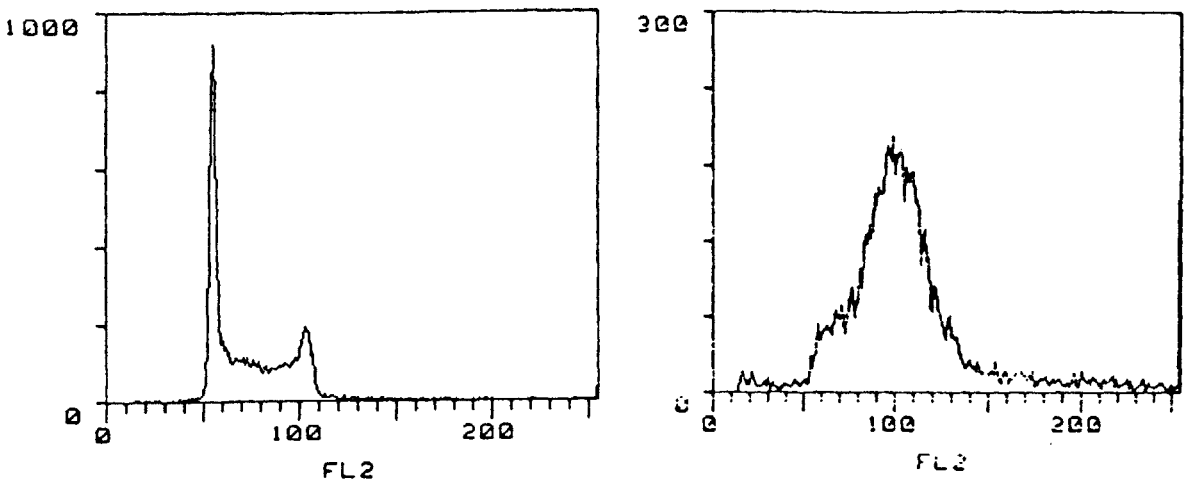


Figure 3.2. ^{3}H-Thymidine Uptake by Mitomycin C Treated CTLL-2 Cells. CTLL-2 cells were incubated in 20 μg/ml of Mitomycin C or in plain media for 2 hours. Cells were then washed and resuspended at a concentration of 10,000 cells/ml in media containing 1 ng/ml of IL-2 or in IL-2 free media. The cells were incubated for 36 hours at which time 0.5 μCi of ^{3}H-thymidine was added to all samples. 24 hours later all samples were harvested and analysed for ^{3}H-thymidine incorporation. Trials were run in duplicate and values represent the means and standard deviations of the data.

positive culture. For both the treated and control cell populations, no appreciable ^{3}H-thymidine uptake was seen when the cells were not cultured with IL-2. From these results we can conclude that Mitomycin C treatment of CTLL-2 cells results in a non-proliferating population whose viability can be preserved for at least several days in IL-2 culture.

To demonstrate that Mitomycin C treatment arrests CTLL-2 cells in the S or M phase of the cell cycle, we performed cell cycle analysis on a population of CTLL-2 that had been treated with Mitomycin C and then cultured in IL-2 containing media for 36 hours. These cells were fixed in ethanol, treated with RNAase, stained with propidium iodide, and analyzed in a fluorescent activated cell scanner (FACS). This analysis would show the distribution of a population according to its cell cycle position, with those cells that are in G_0 or G_1 possessing the minimum amount of DNA and staining in a manner proportional to 2n chromosomes. Cells synthesizing DNA in the S phase of the cell cycle will stain between the 2n and 4n points, and finally cells in G_2 or M phase will yield a peak at the 4n point. Figure 3.3 shows the results of this procedure. Panel A is a histogram which shows the staining pattern for untreated control CTLL-2 cells. The first large peak signifies that most of the cells are in G0 or G1 phase, with a continuum of cells in the S phase and another smaller peak representing the cells in G2 or M phase. This is in contrast to the Mitomycin C treated cells that are shown in panel B. As can be seen, the early peak of cells in G_0 or G_1 is absent as the cells are predominantly in late S, G_2, or M phase. This result is consistent with the expected effect of Mitomycin C.

Cell Cycle Analysis of CTLL-2 Cells Exposed to the Immobilized IL-2 Membrane. Since it has been shown that CTLL-2 cells can be arrested into a non-proliferative state by treatment with Mitomycin C, and that incubation with immobilized IL-2 causes CTLL-2 cells to apparently stop proliferating without loss of viability, we investigated whether cells incubated with immobilized IL-2 are arrested in their cell cycle using propidium iodide staining and FACS analysis. This experiment should clarify if the incubation of CTLL-2



A B

Figure 3.3. Cell Cycle Profiles of CTLL-2 Cells Treated with Mitomycin C. CTLL-2 cells were incubated with 20 mg/ml of Mitomycin C or with plain media for 2 hours and then washed and resuspended in media with IL-2. Thirty six hours later, the cells were removed, fixed, stained with propidium iodide and analyzed in a fluorescent activated cell scanner. The resulting histograms are shown above where (A) cells that were treated with plain media, and (B) cells that were treated with Mitomycin C.

cells with immobilized IL-2 causes cells to become synchronized into one phase of their cell cycle or if the cells simply slow down or stop their proliferative process whatever their position in the cell cycle.

Figure 3.4 shows histograms of propidium iodide staining for cells that have been incubated with the immobilized IL-2 membrane from 0 to 36 hours. Panel A presents the control of stock cells taken from their growth media that represent the T_0 time point for all of these figures. Panel B presents the cells which had incubated on the immobilized IL-2 membrane for 12 hours, panel C for 24 hours, and panel D for 36 hours. As can be seen, the histograms of all of these cases are very similar and there is no marked change in the cell cycle positions for the cells incubated with immobilized IL-2 vis a vis the T_0 control. This same data is presented in a more quantitative form in the column plot of Figure 3.5. In this format, the areas of the G_0, G_1 phases, the S phase, and the G_2, M phases are expressed as a percentage of the total population of cells.

These results indicate that incubating CTLL-2 cells on the immobilized IL-2 membrane does not cause the cells to become synchronized or to accumulate in a given phase of the cell cycle over the 36 hour observation period.

Figure 3.6 presents the cell cycle distributions of several of CTLL-2 control populations. The T_0 cells from the con A maintenance media, cells that have been incubated in IL-2 free media for 12 and 24 hours ((-) controls), and cells that have been incubated with soluble IL-2 for 12, 24, and 36 hours ((+) controls) are shown. While there are no significant differences between the T_0 samples and the negative controls at 12 and 24 hours, the cells that were incubated with soluble IL-2 do have a different profile at 12 and 24 hours. The populations have significantly higher proportions of cells in the S and G_2 or M phases than the negative controls or the initial stock culture.

Because the (+)12 cells demonstrate a significantly different cell cycle profile than the T_0, (-), and immobilized IL-2 samples, we used these cells to check how the immobilized IL-2 membrane arrests cell cycle progression. If the immobilized IL-2

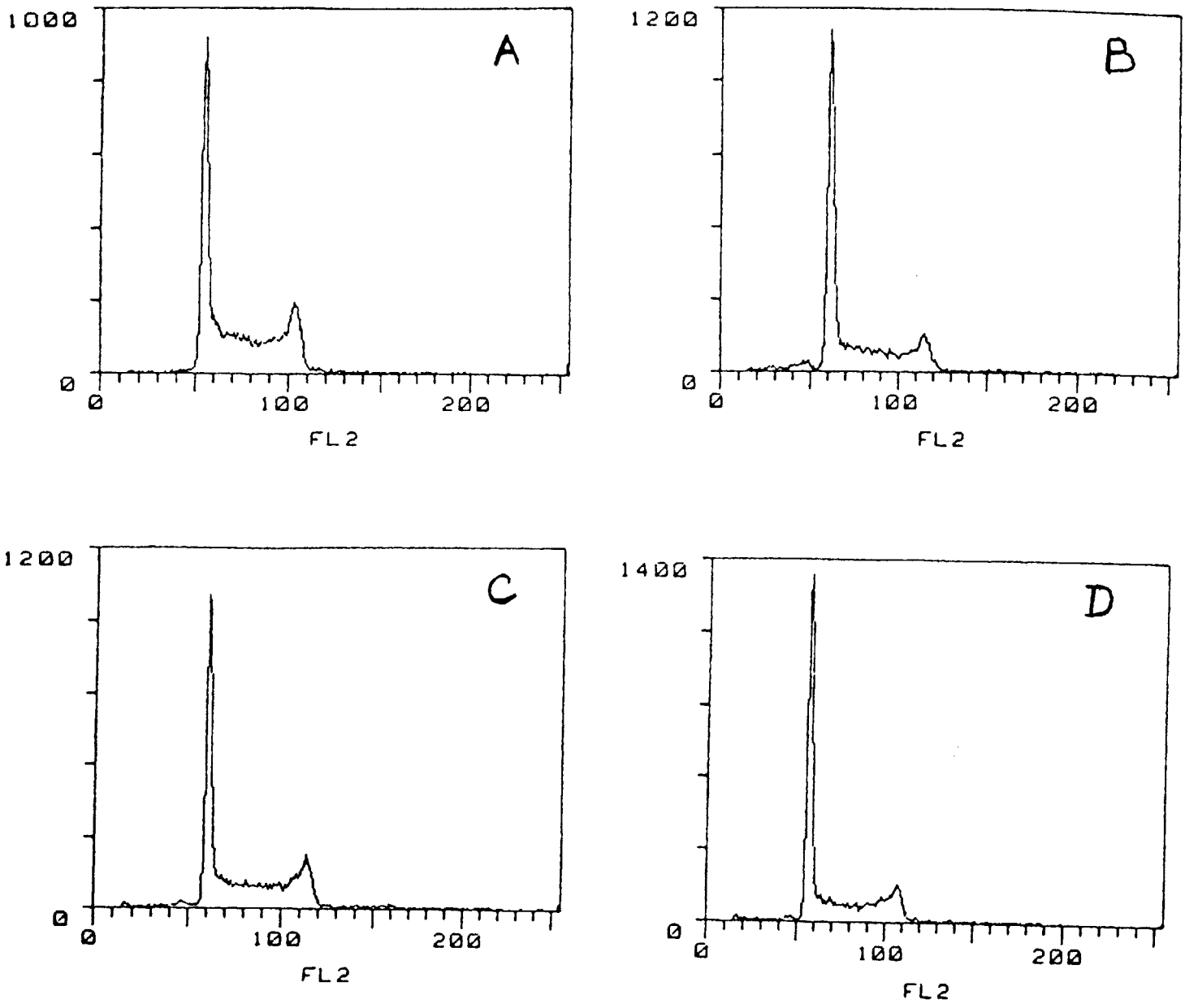


Figure 3.4 Cell Cycle Histograms of CTLL-2 Cells Incubated on the Immobilized IL-2 Membrane. CTLL-2 cells were washed and loaded on top of an immobilized IL-2 membrane. Samples were incubated for various times before they were collected and analyzed by propidium iodide staining and FACS. Panel A shows the resulting histogram for cells collected at the beginning of the experiment. Panels B, C, and D presents the histograms for cells that have been incubated for 12, 24, and 36 hours, respectively, on the immobilized IL-2 membrane. Ten thousand CTLL-2 cells were processed per histogram.

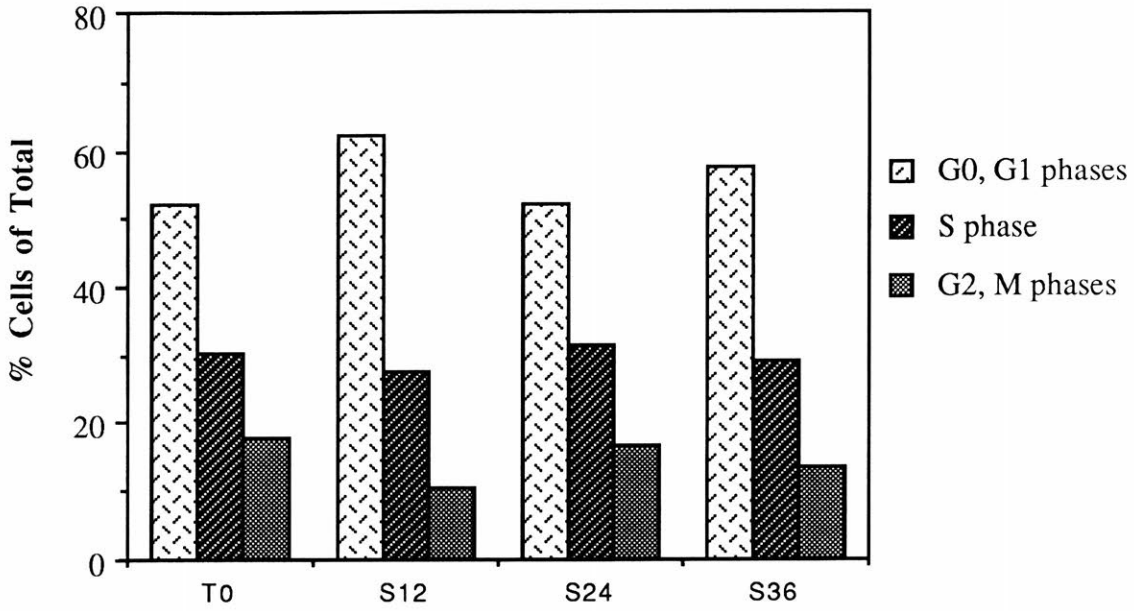


Figure 3.5. Propidium Iodide Staining of CTLL-2 Cells Incubated with the Immobilized IL-2 Membrane. A graphical representation of the histograms presented in Figure 3.4. where the integrated area under the peaks of the G_0 & G_1, S, and G_2 & M phases were determined and are plotted as a percentage of the total number of cells. T0 are sampled taken freshly from the culture media. S12, S24, and S36 refers to samples that have been incubated on the immobilized IL-2 membrane for 12, 24, and 36 hours, respectively.

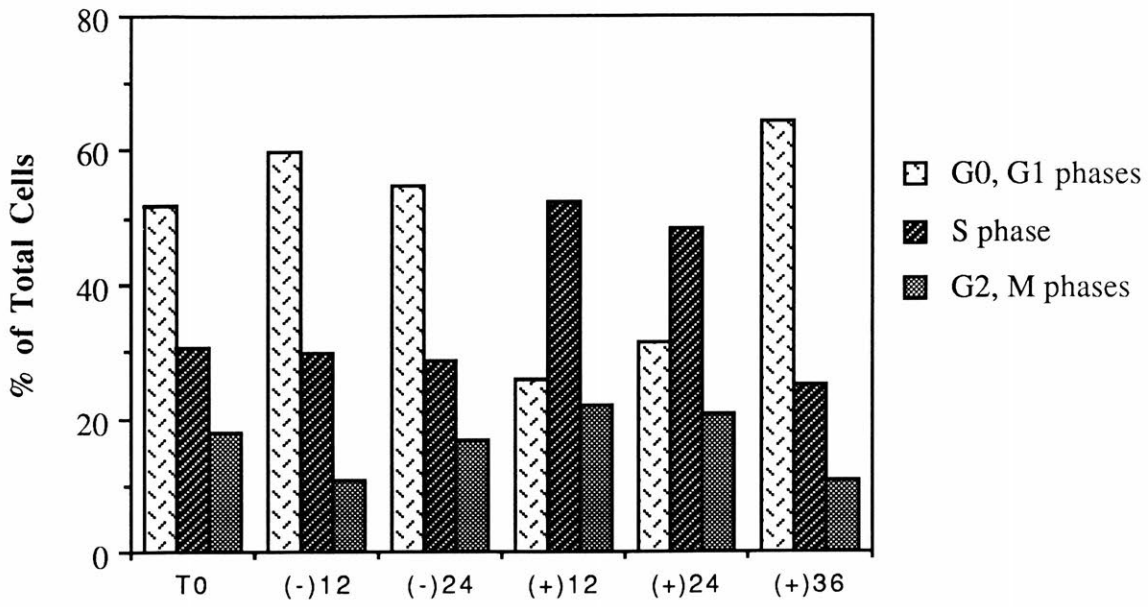


Figure 3.6. Cell Cycle Analysis of CTLL-2 Cell Control Studies. CTLL-2 cells were incubated with soluble IL-2 (1ng/ml) for 12, 24, and 36 hours ((+)12, (+)24, (+)36) or with no IL-2 for 0, 12, and 24 hours (T0, (-)12, (-)24) respectively. The samples were then fixed in ethanol, stained with propidium iodide, and analyzed by FACS to determine their cell cycle distributions. The figures above show the percentages of the total number of cells that are in either the G_0 & G_1 phases, the S phase, or the G_2 & M phases of the cell cycle. Ten thousand cells were analyzed by the FACS scanning per sample.

membrane arrests the population in their initial state when loaded, it would be expected that the (+)12 profile would be maintained after incubation on the membrane. If the membrane works by a different mechanism than directly freezing cells in their cell cycle position, it would be expected that the (+)12 profile would revert to that of the T_0 or (-) controls. Figure 3.7 presents the results of this experiment. Cells that were incubated with soluble IL-2 for twelve hours were washed and placed on the immobilized IL-2 membrane. As can be seen in Figure 3.7 panel A, after the cells were place on the immobilized IL-2 membranes, their cell cycle profiles reverted to that of the distribution seen in the T_0 controls. Panel B of the figure demonstrates that cells incubated in IL-2 free media after the 12 hour pulse of IL-2 will also revert to a profile similar to T_0. Therefore, the immobilized IL-2 membranes do not cause a noticeable synchronization of the population or freeze the population in their initial cell cycle positions.

It should be stressed that although the cell cycle profiles of the cells incubated with the immobilized IL-2 membrane, without any IL-2, and with the T_0 con A supernatant controls have similar cell cycle profiles, morphologically and metabolically these cells are very different. The T_0 samples are a proliferating population where the population is most often in clumps and clusters of rounded cells. The cells incubated with the immobilized IL-2 membrane are a non-proliferating population of adherent and elongated, discrete single cells, and the cells incubated without any IL-2 are granular, rounded, non adherent cells. Thus the cell cycle profile should not be considered indicative as to whether the population is vital or necrotic.

A final investigation using cell cycle analysis involved 12 hour soluble IL-2 treatment of cells that have been incubating with the immobilized IL-2 membrane for 36 hours. From the results of the immobilized IL-2 rescue assays presented in chapter 2 it has been established that cells incubated on the immobilized IL-2 membrane for 36 hours will proliferate in response to exogenous soluble IL-2. We characterized this response in terms of the cell cycle profile for this population both for added soluble recombinant IL-2 and for

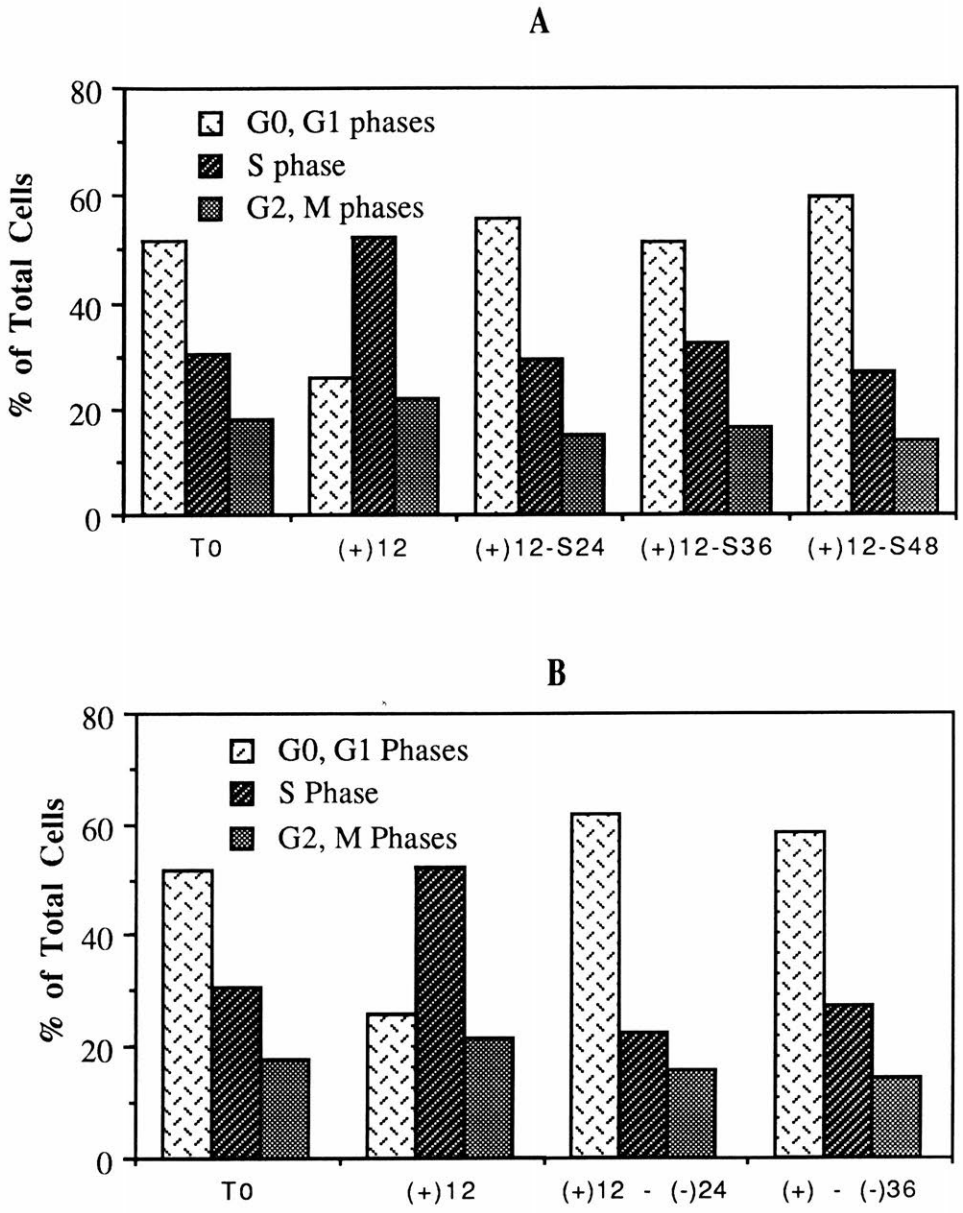


Figure 3.7. Cell Cycle Analysis of CTLL-2 Cells Incubated in Soluble IL-2 for 12 hours and then Incubated with the Immobilized IL-2 Membrane. Panel A) CTLL-2 cells were incubated with soluble IL-2 for 12 hours ((+)12), and then loaded on top of the immobilized IL-2 membrane and incubated for an additional 12, 24, and 36 hours ((+)12-S24, (+)12-S36, (+)12-S48). The control zero time point (T_0)is also shown. Panel B) CTLL-2 cells were incubated with soluble IL-2 for 12 hours ((+)12) and then incubated in IL-2 free media for another 12 ((+)12-(-)24), or 24 hours ((+)12-(-)36). Samples were removed from their incubations, fixed, stained with propidium iodide, and analyzed by FACS to determine their cell cycle positions.

added concanavalin A supernatant that is used in the maintenance medium for CTLL-2 cells. As shown in Figure 3.8, both sources of exogenous IL-2 skewed the cell cycle profiles toward a greater number of cells in S phase. Although this skewing was not as acute as that seen for the (+)12 sample, the trend is similar and indicates that incubation with immobilized IL-2 does not significantly alter the cell cycle response of cells treated with a pulse of exogenous IL-2.

Anti IL-2Rα Staining. As discussed in chapter 1, the p55 α chain of the IL-2 receptor or Tac antigen is transiently expressed in most IL-2 responsive cells and expression levels increase when cells are incubated with IL-2. To determine what effect immobilized IL-2 had on expression levels of this protein, we stained CTLL-2 cells that had been incubating on immobilized IL-2 with a fluorescent labeled rat anti-mouse IL-2Rα antibody. Control histograms are shown in Figure 3.9. Panel A displays the fluorescent staining with the anti-Tac antibody of CTLL-2 cells taken from the concanavalin A supernatant maintenance media and represents the initial expression level for this study. Panel B is a control where the anti-Tac antibody is used to stain P815 mastocytoma cells which do not express the Tac antigen. Panel C displays the staining of CTLL-2 cells by a fluorescent labeled rat anti-mouse IgG antibody. The low staining levels for the controls shown in panels B and C indicate that the staining is specific for the Tac antigen expressed on the CTLL-2 cells.

A statistical analysis of the anti-IL-2Rα stained CTLL-2 cell histograms allows for quantification of the results. Figure 3.10 presents the results of the staining procedure on CTLL-2 cells incubated with soluble IL-2, without any IL-2, and with immobilized IL-2 for different time points. As can be seen, the fluorescent intensities of Tac antigen staining increase by approximately a factor of 2 from initial levels in response to soluble IL-2 in the first twelve hours of incubation and retreat slightly over the following 24 hours, whereas both the cells with no IL-2 and with immobilized IL-2 decline by about factor of 2 in the first 12 hours from initial levels and continue to decline in the ensuing time points. Thus

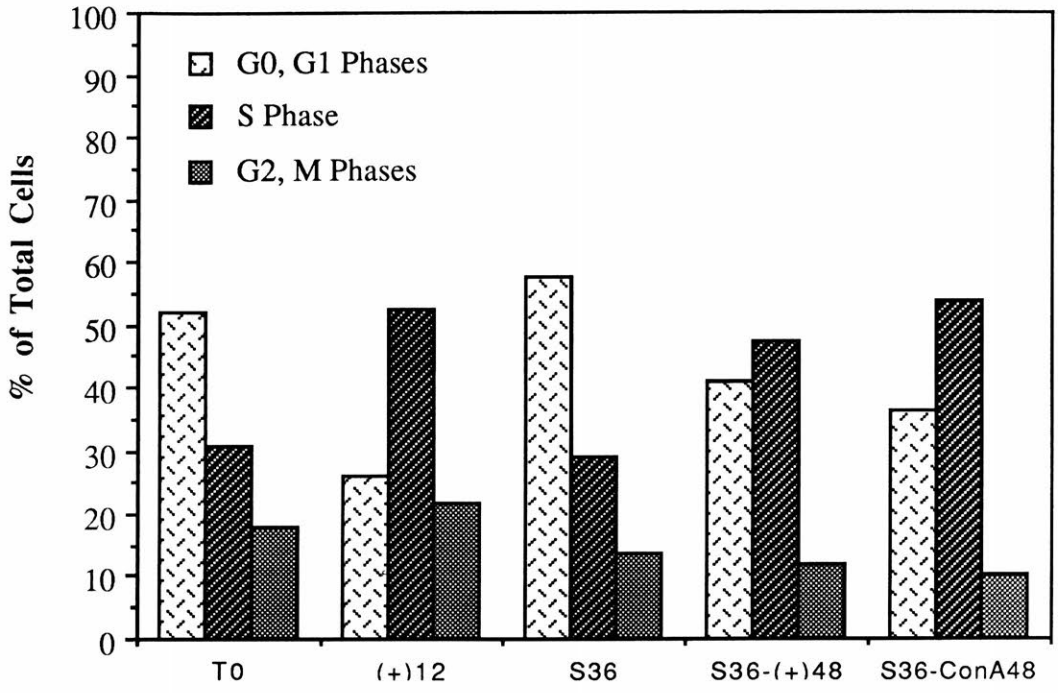


Figure 3.8. Cell Cycle Analysis of CTLL-2 Cells After 36 Hour Incubation with the Immobilized IL-2 Membrane. CTLL-2 cells which were incubated on the immobilized IL-2 membrane for 36 hours were then harvested and replated for 12 hours with either 1ng/ml of soluble IL-2 (S36-(+)48) or the IL-2 rich concanavalin A (S36-ConA48)supernatant media and then processed for propidium iodide staining and cell cycle analysis. Also shown are the cell cycle profiles for cells that have incubated on the immobilized IL-2 membrane for 36 hours only (S36), cells taken from maintenance cultures (T0), and CTLL-2 cells incubated with soluble IL-2 for 12 hours ((+)12).

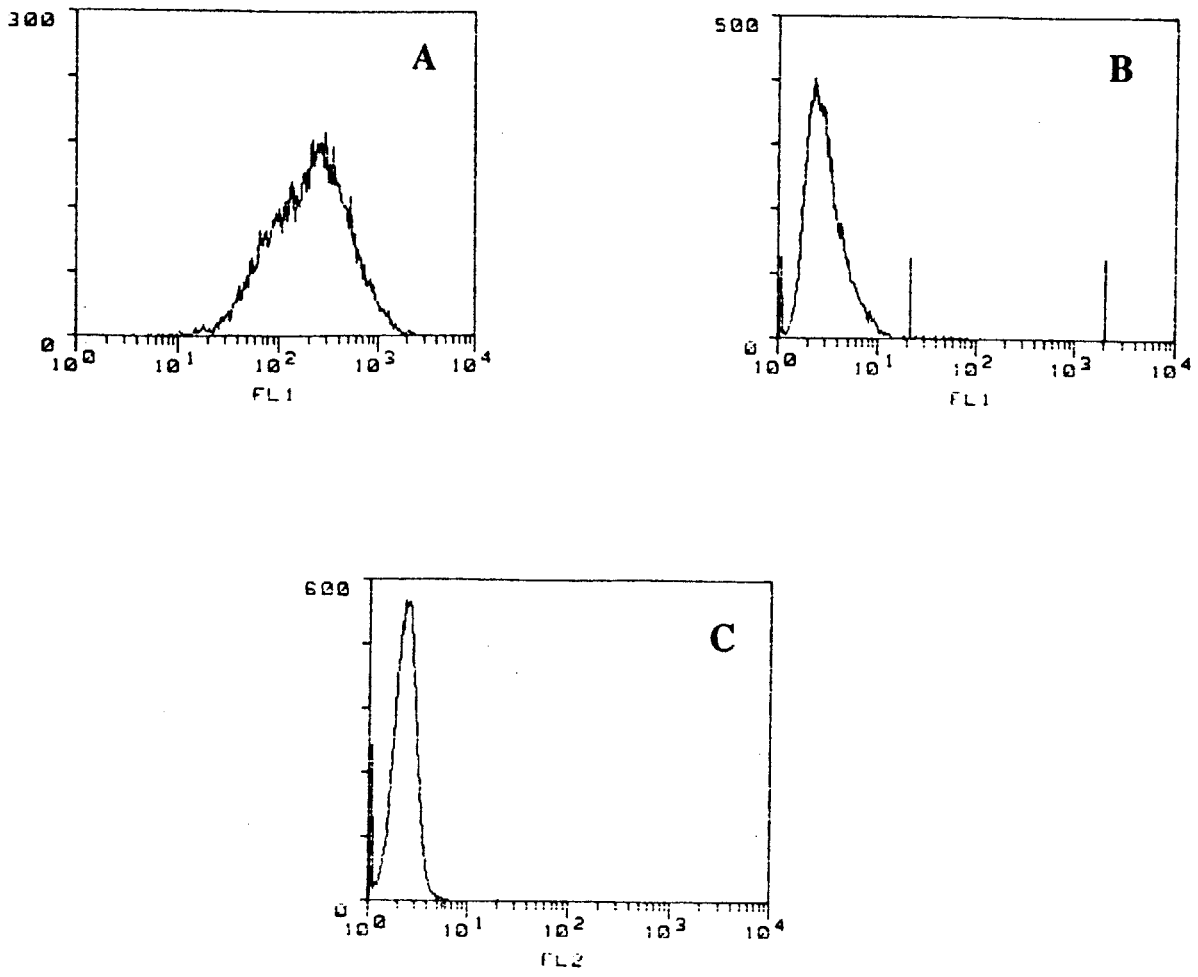


Figure 3.9. Histograms of Anti- IL-2 Receptor α Chain Staining of CTLL-2 Cells and P815 Cells. CTLL-2 cells or P815 mastocytoma cells were taken from maintenance media, washed, blocked with normal rat serum, and stained with fluorescein labeled rat anti-mouse anti-IL-2R α chain antibody. Panel A presents the histogram of the CTLL-2 cells that were stained with the anti-IL-2R α antibody. Panel B shows the response for P815 cells that are stained with the same anti-body and panel C presents CTLL-2 cells stained with an irrelevant rhodamine labeled rat anti-mouse IgG monoclonal antibody.

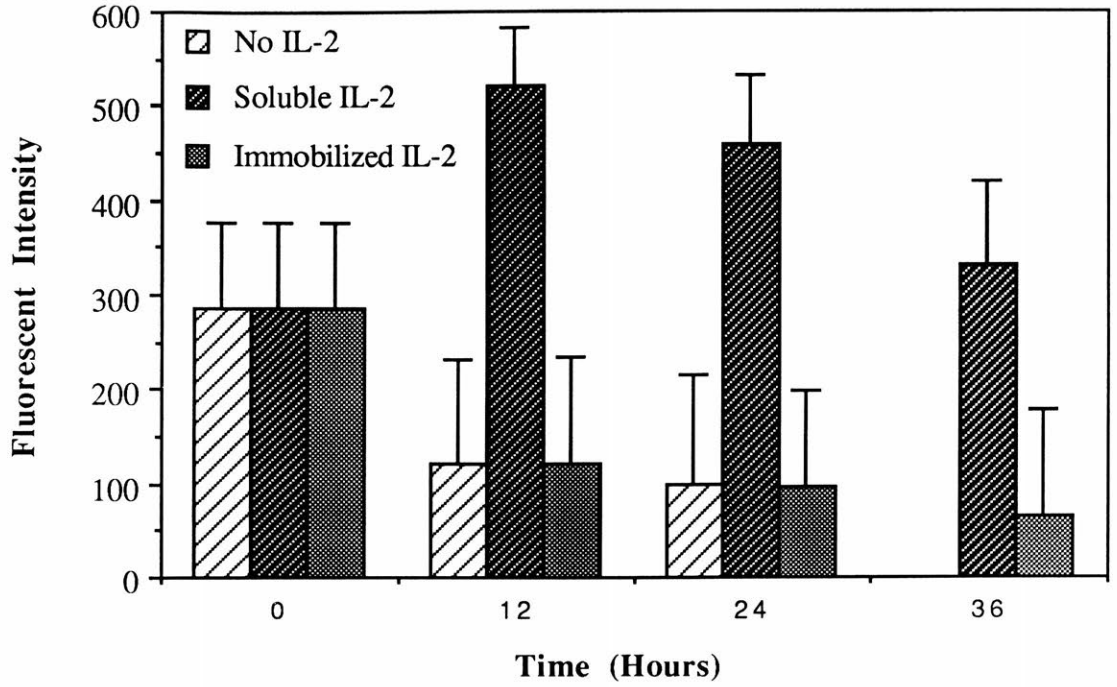


Figure 3.10. Anti-IL-2 Receptor α Chain Staining of CTLL-2 Cells Incubated on an Immobilized IL-2 Membrane. CTLL-2 cells were incubated on top of an immobilized IL-2 membrane for 12, 24, and 36 hours, at which time they were stained with a fluorescein labeled rat anti-mouse IL-2R α chain monoclonal antibody. The results are depicted above along with control studies where CTLL-2 cells were incubated with soluble IL-2 (1ng/ml) for 12, 24, and 36 hours and without any IL-2 for 12 and 24 hours. The mean fluorescent intensity and standard deviations are given for the 10,000 cells analyzed by FACS for each sample.

the immobilized IL-2 membrane does not elevate the expression of the IL-2Rα chain as does soluble IL-2.

Since exposure of CTLL-2 cells to soluble IL-2 (1 ng/ml) can elevate the expression level of the Tac antigen significantly over background, we checked to see whether immobilized IL-2 was capable of preserving these elevated levels. CTLL-2 cells that had been incubated in soluble IL-2 for 12 hours were then washed and incubated either without any IL-2 or with immobilized IL-2. The resulting fluorescent levels are given in Figure 3.11. As can be seen, the immobilized IL-2 membrane does not preserve the expression level of the Tac antigen any more efficiently than incubation of the cells with no IL-2.

This is not to suggest, however, that the cells incubated with the immobilized IL-2 membrane and the cells incubated without any IL-2 are similar populations. There were roughly four fold more viable cells in the immobilized IL-2 cultures than there were in the cultures without any IL-2 after 24 hours where both cultures started with the same number of cells. As mentioned above, the morphology and appearance of these populations also sharply differed.

We also determined whether CTLL-2 cells that had been incubated on the immobilized IL-2 membrane behave in a similar fashion to untreated cells in terms of their expression of IL-2Rα when pulsed with IL-2. CTLL-2 cells that had been incubated on an immobilized IL-2 membrane for 36 hours were then pulsed with soluble IL-2 (1 ng/ml) or concanavalin A supernatant media and assayed for their IL-2Rα response after 12 hours. These values are given in Figure 3.12. As shown, the immobilized IL-2 membrane does not change the ability of CTLL-2 cells to respond to exogenous IL-2 sources with heightened expression of the Tac antigen.

Kinetic Analysis of CTLL-2 Cell Response to Soluble IL-2 from Cells Exposed to Immobilized IL-2 Membrane. CTLL-2 cells are most commonly used as a bioassay line for

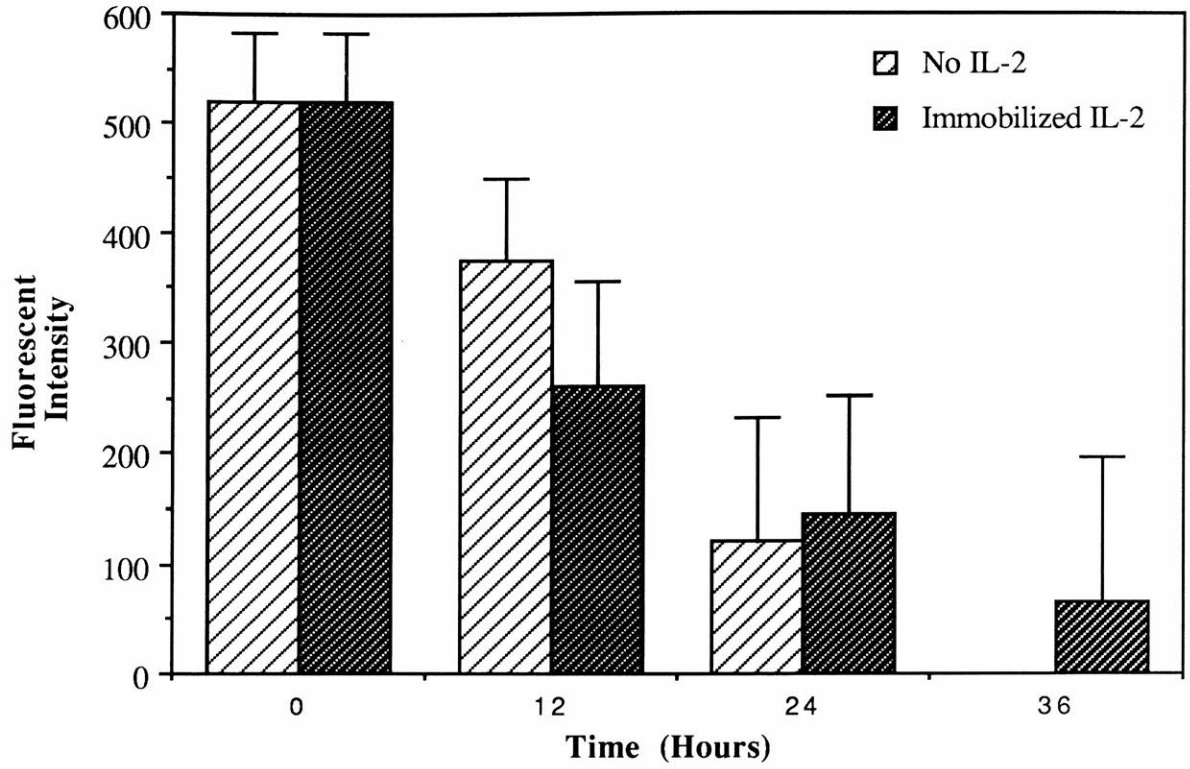


Figure 3.11. Anti-IL-2 Receptor α Chain Staining of CTLL-2 Cells Treated with Soluble IL-2 for 12 hours and then Incubated with Immobilzed IL-2 or without IL-2. CTLL-2 cells were incubated for 12 hours with soluble IL-2 (1 ng/ml) for 12 hours then washed and replated either with no IL-2 or with immobilized IL-2. Samples were harvested at the times shown and stained with a fluorescently labeled anti-IL-2 receptor α chain antibody. The mean fluorescent intensity and standard deviations are given for the 10,000 cells analyzed by FACS for each sample.

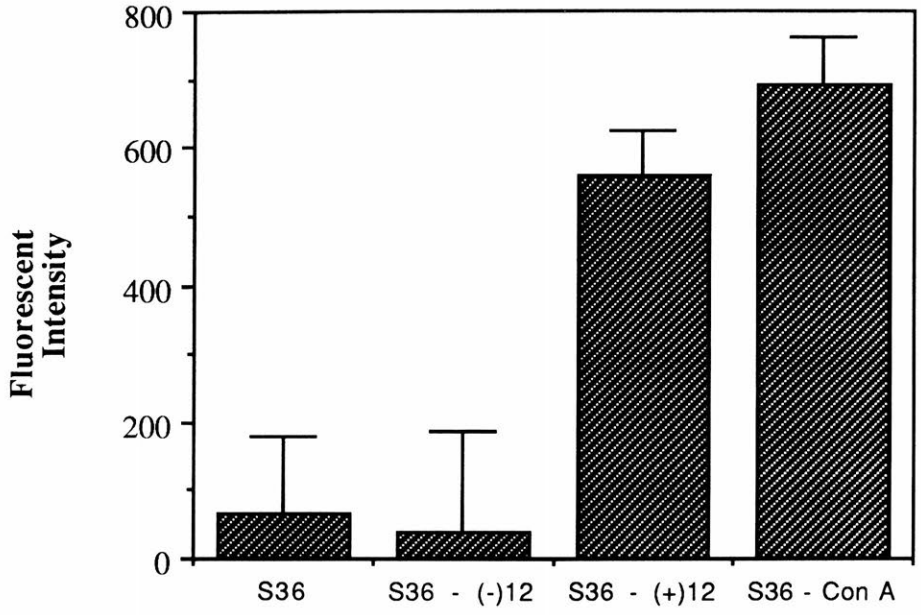


Figure 3.12. Anti-IL-2 Receptor α Chain Staining of CTLL-2 Cells Incubated with Immobilized IL-2 and Then Transferred to Other Culture Conditions. CTLL-2 cells were incubated on top of an immobilized IL-2 membrane for 36 hours and then replated for 12 hours either with IL-2 free media (S36-(-)12), with soluble IL-2 (1 ng/ml) (S36-(+)12), or with the IL-2 containing con A supernatant (S36-Con A). The samples were then harvested, stained with a fluorescently labeled anti-IL-2 α chain monoclonal antibody, and analyzed by FACS. The mean fluorescent intensity and the standard deviations are given for the 10,000 cells analyzed per sample.

the presence of IL-2. This is because they have a reproducible growth response and ^{3}H-thymidine incorporation rate to varying amounts of IL-2 that is generally linear for IL-2 concentrations in the low picomolar range. To determine whether incubation with immobilized IL-2 alters the behavior of CTLL-2 in response to soluble IL-2, we assayed the ^{3}H-thymidine incorporation in an IL-2 titration of CTLL-2 cells that had been preincubated with soluble IL-2, with no IL-2, and with immobilized IL-2. A sample curve is presented in Figure 3.13 for cells which had been incubated in soluble IL-2 (1 ng/ml) for 12 hours before being washed and titrated with IL-2.

A rectangular hyperbola was fitted to the titration curves of CTLL-2 cells from different conditions to arrive at quantitative parameters to characterize their behavior . The parameter Km is defined as being the value of IL-2 concentration at which the cellular response is exactly half of the theoretical maximum. The higher the Km value, therefore, the lower the sensitivity of the cells for IL-2. The parameter Vmax is defined as the theoretical maximum response that is approached as the IL-2 concentration reaches saturating conditions. The Vmax values, normalized to a basis of 3H-thymidine incorporated per viable cell loaded, were not significantly different between any experiments. As shown in Table 3.1, there are also no significant differences between the kinetic parameters Km for the cases where CTLL-2 cells were incubated without any IL-2 and where the cells were incubated with immobilized IL-2. However, the cells incubated with soluble IL-2 showed a steady increase in their Km values over the 36 hours.

3.4. Discussion

It was established in the previous chapter that an immobilized IL-2 membrane is capable of preserving the viability of the mouse T cell line CTLL-2, a cell type that is dependent on IL-2 for both growth and viability. In this section, studies were presented that sought to explain how CTLL-2 cells are afftected by the immobilized IL-2 membrane.

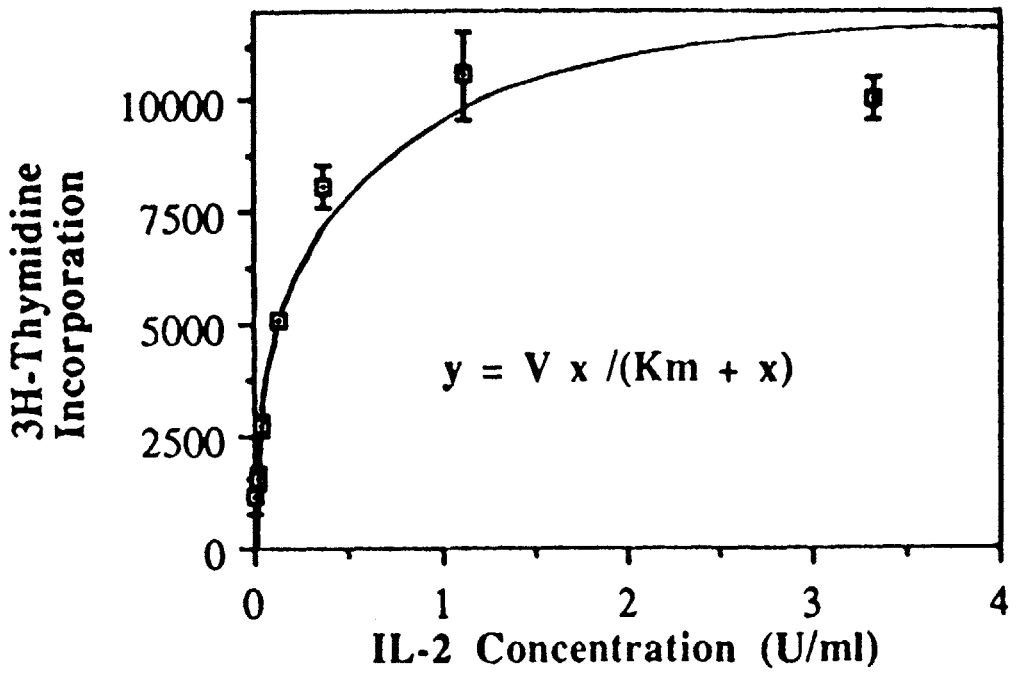


Figure 3.13. Sample IL-2 Titration Curve. CTLL-2 cells that had been incubated with soluble IL-2 for 12 hours (1ng/ml) were washed and replated with the amounts of IL-2 shown. After 18 hours, 0.5 microcuries of tritiated thymidine was added to all wells and 24 hours later the samples were harvested and assayed by scintillation counting. A curve of the form shown above was fitted to the data to determine the Km value for the titration.

Table 3.1. K_m Values for IL-2 Titration Curves by CTLL-2 Cells Pre-incubated Under Different Conditions§.

Time (hours)	(+) Control* Values (ng IL-2/ml)	(-) Control ** Values (ng IL-2/ml)	Immobilized IL-2*** Values (ng IL-2/ml)
12	.0543	.0423	.0430
24	.1120	.0303	.0270
36	.1676	xxxxx¶	.0343

§CTLL-2 cells were incubated in the manners described above, then washed and replated in varying amounts of IL-2 in the wells of a 96 well culture plate. Twelve hours after the replating, 0.5 μCi of ^{3}H-thymidine was added to all of the wells, and 24 hours after the replating the samples were analyzed for ^{3}H-thymidine uptake. Background values were subtracted from the experimental values, and a curve was fit to the resulting data of the form :

$$\text{Uptake (DPM)} = V \times [\text{IL-2}] / (K_m + [\text{IL-2}])$$

These K_m values, which are a measure of the sensitivity of the cells response to IL-2, are shown above.

* CTLL-2 cells were incubated in 1ng/ml of IL-2 for the indicated times, washed in plain media, and resuspended in varying amounts of IL-2 to generate the IL-2 response curve.

**CTLL-2 cells were washed and incubated in IL-2 free media for the indicated times, then washed again and resuspended in varying amounts of IL-2 to generate the IL-2 response curve.

***CTLL-2 cells were washed and plated on top of immobilized IL-2 membranes for the times indicated. The cells were then eluted from the membranes by treatment with 5% DMSO and .05 mM EDTA, rinsed twice, and replated with varying amounts of IL-2 to generate the IL-2 response curve.

¶There were no viable cells remaining when CTLL-2 cells were incubated without IL-2 for 36 hours.

By characterizing different IL-2 mediated responses in CTLL-2 cells, it was possible to arrive at a much clearer picture of the state of cells exposed to immobilized IL-2, and determine whether the immobilized IL-2 membrane alters any fundamental properties of CTLL-2 cells.

The section on Mitomycin C treatment of CTLL-2 cells presents clear evidence that the cells require IL-2 to maintain their viability even when these cells are not proliferating. This is the first time that preservation of viability and growth induction have been shown to be distinct activities of IL-2 on CTLL-2 cells. The propidium iodide staining examines how a population of CTLL-2 cells progress through their cell cycle when they are stimulated with soluble IL-2 and when they are dying from IL-2 deprivation and relates these results to the cell cycle progression from exposure of the cells to immobilized IL-2. Likewise, staining the cells for the IL-2Rα chain follows the course of expression of an activation antigen that is stimulated by IL-2 and allows characterization of the effectiveness of the immobilized IL-2 membrane in transmitting IL-2 specific signals. Finally, evaluating the kinetic profile of CTLL-2 growth response to soluble IL-2 as a function of cell history examines changes in sensitivity of the cells to IL-2.

The portrait that emerges from these studies is that CTLL-2 cells which are incubated with the immobilized IL-2 membrane phenotypically resemble the IL-2 deprived control populations and are essentially unchanged in their properties by this incubation. The immobilized IL-2 membrane did not detectably cause any deviations from the behavior patterns defined by the control populations of cells incubated with and without soluble IL-2. Propidium iodide staining of the cells demonstrated that the cells incubated on the immobilized IL-2 membrane and cell incubated without any IL-2 have cell cycle profiles that very closely resemble one another. In addition, IL-2R α chain staining demonstrated that expression levels of this antigen are very similar between these two cell populations. It was also shown that CTLL-2 cells that were incubated on an immobilized IL-2 membrane and CTLL-2 cells that were incubated without any IL-2 had essentially the same response

to the addition of soluble IL-2 as assayed by the staining techniques used above or by characterizing the kinetic parameters of the proliferative response of the cells to the exogenous IL-2. These phenotypic characteristics are summarized for comparison in Table 3.2 for the cell populations that have been incubating 24 hours in soluble IL-2, with immobilized IL-2, and with no IL-2, respectively.

The result that the CTLL-2 cells which are incubated on the immobilized IL-2 membrane are phenotypically similar to the cells which are incubated without any IL-2 is surprising in light of the morphological differences between these populations. The cells which are incubating with the immobilized IL-2 membrane are adherent and elongated cells that also appear to be agranular with distinct cellular and nuclear membranes when they are examined by phase contrast microscopy. The cells which are incubated without any IL-2 are nonadherent and rounded, with increasing granularity in their cytoplasms irregularity in their cellular membranes as one observes them 12 to 24 hours after seeding. The viability of these populations have been shown to be very disparate, with only about 30% of the IL-2 deprived cells viable after 24 hours whereas the cells incubating on the immobilized IL-2 membrane have greater than 70% viability after 36 hours. This point is illustrated in Table 3.3, where viable cell counts for CTLL-2 cells which have been incubated with soluble IL-2, immobilized IL-2, and no IL-2 for varying times are presented. As shown, the viable cell counts for the population incubated with no IL-2 fall off drastically after 12 hours while the samples incubated with immobilized IL-2 preserve their viability. Thus, morphologically and in terms of their prospective viabilities, CTLL-2 cells that are incubated with an immobilized IL-2 membrane and cells that are incubated without any IL-2 are very different in spite of their phenotypic similarites .

It is apparent that the immobilized IL-2 membrane provides some function that is required to maintain the viability of CTLL-2 cells, but that this function is not comparable to soluble IL-2. Roughly, there are three possible explanations, the specific, non-specific, and a combination of the two. By non-specific, it is meant that the phenomenon is not due

Table 3.2. Comparison of Cell Characterization Studies for 24 hour Samples.

Category	CTLL-2 Cells Incubated for 24 hours with Soluble IL-2 (1 ng/ml)	Immobilized IL-2	No IL-2
Morphology*	Large, rounded non-adherent	Elongated, adherent	round, non-adherent granuluar
IL-2 R α chain expression (fluorescent intensity)	458 $\pm$ 73	97 $\pm$ 100	99 $\pm$ 110
Relative Size (Forward Light Scatter)	88.4 + 17	74 $\pm$ 23	84 $\pm$ 24
Cell Cycle Analysis			
% G_0, G_1	31	52	55
% S	48	31	28
% M, G_2	21	17	17
Sensitivity to IL-2 (K_m -ng/ml)	0.112	0.027	0.030

*Observed by phase contrast microscopy.

Table 3.3. Viable Cell Counts of Samples.*

Incubation Time (hr)	Soluble IL-2 (1 ng/ml)	Immobilized IL-2	No IL-2
0	40,000	40,000	40,000
12	60,000	50,000	50,000
24	110,000	40,000	15,000
36	>150,000	40,000	<5,000

*CTLL-2 were plated at 40,000 cells per well at time zero in 1 ml of media and either soluble IL-2 (1 ng/ml), immobilized IL-2, or no IL-2. Samples were harvested at 12 hour intervals, washed in media, resuspended and centrifuged at 10 x g for 10 minutes. The samples were then resuspended and an aliquot was analyzed by trypan blue staining for viable cells. The remaining cells were used for the propidium iodide staining procedures.

to specific signalling events from the binding of immobilized IL-2 and the IL-2 receptors on the incubating cells. This explanation would invoke the fact that the CTLL-2 cells become adherent to the membrane and are more elongated, whereas cells in the IL-2 free media are uniformly rounded, especially after 18 hours when they begin to die. It may that since the cells are 'stuck' and elongated, they are somehow prevented from making the cytoskeletal and metabolic changes that lead to cell death. An experiment that might demonstrate this hypothesis would be to incubate CTLL-2 cells on a surface to which they would adhere, such as polylysine, in an IL-2 free media and then to examine viability after 36 hours. Yet this experiment could only possibly demonstrate a phenomenon. A real understanding of the process would require an extensive analysis of how the cells and matrix interact and what cell membrane proteins are involved.

The specific explanations for the phenotypic and functional character of the cells incubated on the immobilized IL-2 membrane would suppose that some specific signalling does take place from the interaction of the immobilized IL-2 and the IL-2 receptors on the CTLL-2 cells. Yet this interaction does not mimic high concentrations of soluble IL-2. The two possibilities that account for this is that either the immobilized IL-2 does not behave like soluble IL-2 in terms of receptor signalling, or that immobilized IL-2 is like soluble IL-2 only the ideosyncrasies of our system cause the effective concentration of IL-2 to be very low so that the IL-2 stimulation is vanishingly slight. The second case would be analogous to the presence of trace amounts of soluble IL-2, enough to keep the cells alive but not enough to stimulate detectable growth. This scenario may be judged to be unlikely if only because we have observed in extensive numbers of IL-2 titrations that as the concentration of IL-2 is reduced, more and more cells die, but those cells that remain viable also appear to be growing due to the presence of viable cell pairs and clusters. The population has not been observed to uniformly stop growing. The first case mentioned above where immobilized IL-2 acts differently than soluble IL-2 would suggest that the receptor needs to be free to diffuse along the cell surface and to be internalized in order to

completely transmit the IL-2 signal, but that just by binding to IL-2 some elements are communicated. The final explanation which is a combination of the specific and non-specific is that there might be no specific signalling from the interaction of IL-2 and the IL-2 receptor, but that it is important that this receptor is the molecule via which cells are bound and adhere. A scenario which falls into this category is if receptor clustering were important for signal transduction. In such a case, the immobilized IL-2 membrane could focus receptors to the portion of the cell membrane contiguous to the immobilized IL-2 surface, but the concentration of IL-2 might not be high enough to allow the receptors to form tight clusters. Therefore the signalling might be intermittant or ineffectual depending on the gyrations of the cell and random processes. A final possibility is the opposite of the non-specific case mentioned above. In this scenario, the receptors bound to the immobilized IL-2 are perfectly active in signalling, but the adherence of the cells to the membrane prevents some other component of cellular response that is further downstream in the IL-2 stimulated signalling cascade. The resolution of these differing possibilities must await the development of an experimental system where the active surface concentration and spatial distribution of IL-2 can be known.

Another phenomenon which was uncovered in these studies was the distinctive behavior of the (+)12 control cell populations. CTLL-2 cells grow vigorously in the concanavalin A media and have a doubling time of about 15 hours. Yet samples taken from this media and treated with 1 ng/ ml of soluble IL-2 responded with a drastically increased level of IL-2 receptor α chain expression and with a cell cycle profile that was significantly skewed toward the S phase. The fact that soluble IL-2 can so markedly change these indicators from the con A controls also relates something about the manner in which IL-2 stimulates a population.

CTLL-2 cells will manifest a growth response to soluble IL-2 in a fashion consistent with a saturation kinetics model over a range from roughly 0.01 ng/ml to 1.0 ng/ml of IL-2. This means that over the lower part of the range, the cellular response is

linearly proportional to the IL-2 concentration and over the upper part some maximal level of response is approached. There have long been two different explanations for this phenomenon. The first is that for a growing population, all cells are proliferating and each individual cell has a spectrum of growth rates; thus a cell responds to increasing IL-2 by increasing that growth rate. The second explanation is that cells have a fixed growth rate, and the increased response of a population of cells to IL-2 is a result of increasing the number of cells that are proliferating. The results of these studies demonstrate a phenomenon that is consistent with this second explanation. The cell cycle profiles for the T_0 con A samples and the (+)12 samples shown in Figure 3.6 provide a case in point. If the increased amounts of IL-2 caused a uniform increase in proliferation rate among all cells, the cell cycle profile for (+)12 would mimic that seen for T_0 as each individual cell simply increased its growth rate and the distribution of all cells remained uniform. Instead, we see that the addition of the soluble IL-2 caused a skewing of the population into the S phase. This can be explained if the sudden increase of IL-2 at time zero recruited a disproportionate share of cells to enter the cell cycle at that time. The diminution of the size of the S phase peak for the (+)24 and (+)36 hour samples may represent either the gradual loss of the pulse due to random processes, an intact pulse aligning with different phases of the cell cycle when the samples were taken, or it could be that the initial stimulation of CTLL-2 cells by the IL-2 pulse is a transient response of the cells that is not maintained even when the IL-2 concentration is preserved. The behavior probably does not represent reduction of cell proliferation due to depletion of media components or IL-2 since the initial seeding density of cells was low.

Another piece of evidence for our interpretation that the pulse of IL-2 works to recruit quiescent cells into a stimulated state that might be transient comes from the anti-IL-2R α chain staining experiments. As depicted in panel A of Figure 3.13, the stained concanavalin A maintained control cells range in fluorescent intensity from about 20 to 2000 and have a mean value of 286. The (+)12 samples seen in panel B also range from

20 to 2000 but have a mean value of 521. Thus rather than the population uniformly shifting, the cells behave as if their are two distinct populations, lows and highs. The lows, with a mean fluorescent intensity about 100, are shifted to the highs, with a mean intensity of about 500, for the (+)12 sample. The (+)24 and (+)36 hour samples shown in panels C and D illustrate how the highs shift back toward the lows with increasing time. Thus the staining supports the interpretation that cells move from discrete unstimulated to stimulated states with the pulse of soluble IL-2 and that this behavior may be transient.

In conclusion, the experimental studies presented in this chapter were successful in two significant respects. These studies were able to answer important unresolved questions about the activity of immobilized IL-2 membrane and how it would effect CTLL-2 cells. These results demonstrate that the membrane does not alter cell properties beyond a normal range and that the use of this system could be appropriate as an IL-2 substitute for long term culture or as an affinity matrix for cells expressing the IL-2 receptor without fear that the membrane will adversely affect the cell population. These studies also served to elucidate interesting aspects of the response of a population of cells to a stimulus. It has been seen that the population of CTLL-2 cells is not homogeneous in its response to a stimulus, that the response can be transient even when the stimulus is present, and that cells which are phenotypically and kinetically very similar in their response may be very different in their morphology and prospective viability.

3.5. References

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CHAPTER 4

MODEL OF IL-2 RECEPTOR EXPRESSION

4.1. Introduction

IL-2 mediates its effects by binding to specific IL-2 receptors that are expressed on the surface of IL-2 sensitive cells. These receptors have three distinct forms that bind to IL-2 with differing affinities. The low affinity receptor consists of a single 55 kilodalton glycoprotein that binds to IL-2 with a dissociation constant of 30-100 nM. This protein is also called the α chain of the receptor or the Tac antigen and it has been shown that this molecule does not cause intracellular signalling when it binds to IL-2. The intermediate affinity IL-2 receptor consists of at least two proteins, one of 75 kilodaltons that binds to IL-2 with a dissociation constant of 10 nM that is called the β chain and another of 110 kilodaltons called the γ chain, which may not bind to IL-2 but which is needed for the active signal transduction by this receptor. These two receptors, the low affinity α chain and the intermediate affinity $\beta\,\gamma$ receptor complex, or the β chain for short, can combine to form a third IL-2 binding moiety called the high affinity receptor which has a dissociation constant of 30 pM. These receptors have been the subject of study for many years and much is known about the physical and chemical qualities of these molecules and about the IL-2 receptor expression levels under different environments.

In the preceding chapters of this study, the behavior of the IL-2 sensitive cell line CTLL-2 has been examined under varying conditions of IL-2 presentation. The focus has been to quantify and understand the effects that immobilized IL-2 has on these cells. The use of immobilized IL-2 present a novel system to the CTLL-2 cells. When soluble IL-2 is presented to these cells, the IL-2 is bound by their receptors, and then these ligand-receptor complexes migrate along the cell surface until they reach sites where they are internalized.

The use of immobilized IL-2 is believed to block the free migration and internalization of the ligand-receptor complex while still allowing the receptors to bind. Yet it has been found that an immobilized IL-2 membrane does have a biological effect on CTLL-2 cells. Cells incubating on the immobilized IL-2 membrane maintain their viability over a 36 hour period, where cells incubating without any IL-2 die.

The mechanism by which immobilized IL-2 mediates this preservation of viability has been explored in different studies in the previous chapter. One possibility was that the immobilized IL-2 membrane could work by arresting cells in their proliferative cycle; that by binding and freezing IL-2 receptors to the surface of the cell, the cell was prevented from progressing to mitosis. This hypothesis was disproved by a set of propidium iodide staining experiments presented in Chapter 3 which demonstrated that the cells incubating on immobilized IL-2 are not arrested in their cell cycles. Another possibility was that the immobilized IL-2 could only transmit part of the signal ordinarily mediated by soluble IL-2. Thus cells might receive enough signal to remain viable but not enough for proliferation. Another alternative is that the immobilized IL-2 is capable of mediating activity in the same manner as soluble IL-2, but that the amount of active IL-2 on the surface is insufficient for stimulation.

To explore the differences between these last two possibilities, it would be useful to know how much active IL-2 is present on the surface of the immobilized IL-2 membrane. Although this value could not be measured by experimental means, it might be possible to arrive indirectly at this figure by using some of the data acquired on IL-2 receptor expression and sensitivity to IL-2 by cells incubating on the immobilized IL-2 membrane.

Experiments have been performed that follow the expression levels of the IL-2 receptor α chain and that determine the sensitivity of the cells to soluble IL-2 after different modes of IL-2 exposure. Since these investigations focus mainly on IL-2 receptor behavior, it would be informative to examine the results in light of what is known about the physico-chemical properties of the IL-2 receptors. These properties include the affinities of

the receptors for IL-2, the expression levels of the receptors, and kinetic rates for internalization and externalization of the receptors. The purpose for this examination is to determine whether the immobilized IL-2 mediated behavior can be predicted from elements of prior characterizations of the IL-2 receptor system, and conversely, to gain further insight into the properties of the immobilized IL-2 membrane and of the responding cells from the immobilized IL-2 studies.

For the purposes of this study then, it is necessary to gather and unify diverse elements of the literature to characterize IL-2 receptor behavior. This was accomplished by constructing a mathematical model which incorporated equilibrium and kinetic data of the IL-2 receptor system. The model is used to predict receptor expression levels of all of the different forms of the IL-2 receptor for soluble IL-2 systems, and these predictions are checked by comparison to many of the control studies in previously discussed experiments. The model is applied to gather insight into what aspects of the observed behavior in the immobilized IL-2 incubations can be explained solely by equilibrium and kinetic considerations, and which phenomena require assumptions about the regulatory behavior of the cells. The model is also used to estimate the number of active IL-2 molecules that might be available to a cell incubating on the immobilized IL-2 membrane.

4.2. Model Construction

The current understanding of the physico-chemical properties of the IL-2 receptor system can be summarized under four topics: surface combinations of receptors; IL-2 binding to receptors; receptor internalization; and receptor externalization. Each subject is treated separately below and the values for rate constants are summarized in Table 4.1.

Table 4.1. Model Rate Constants[§].

Parameter	Value (sec^{-1})	Comment/Reference
$k_{\alpha I}$	.00019	Half-life of unbound
$k_{\beta I}$	.00019	receptors is 1 hour (2, 3)
k_{HI}	.00019	
$k_{\alpha^* I}$	.00019	Not affected by binding
$k_{\beta^* I}$	.00077	Half-life of bound receptors
$k_{H^* I}$	.00077	is 15 minutes (2, 3)
k_{α^*}	.05[†]	K_d = 100 nM (4, 5)
k_{β^*}	.0045[†]	K_d = 5 nM (4, 5)
k_{H^*}	.015[†]	K_d = 30 pM (4, 5)
$k_{-\alpha^*}$	3.3×10^{-4}	Reverse binding
$k_{-\beta^*}$	1.5×10^{-4}	kinetics given in
k_{-H^*}	3.8×10^{-5}	(4)
k_H	2×10^{-6}	Tested parameters
k_{-H}	2×10^{-3}	
$k_{\alpha^* H}$	3.5×10^{-4}	Bimolecular surface reaction,
$k_{\beta^* H}$	1×10^{-5}	Diffusion limited
$k_{-\alpha^* H}$	4×10^{-6}	Low probability reactions
$k_{-\beta^* H}$	2×10^{-6}	(1, 6)
$k_{\alpha S}$	1.4, 3.5, or 7	Responsive to [IL-2] (2, 3, 5)
$k_{\beta S}$	3	Constituitive (2, 3, 5)

Table 4.1. Continued

§The values given in this table are the rate constants used in the receptor balance equation of the model. Changes in the values for the simulations of immobilized IL-2 incubations are discussed in the results section for those trials and in the discussion section.

† The values presented are the inherent rate constants unscaled by the affinity of the receptor or the local IL-2 concentration. The scaled values, used in the model, take the form:

$$k_{scaled} = k_{inherent} \times ([\text{IL-2 concentration}] / Kd + [\text{IL-2}]).$$

4.2.1. Surface Reactions of Receptors

As discussed above, the IL-2 receptor system includes two distinct IL-2 binding chains of lower affinities, α and β, which can combine to yield a third higher affinity receptor, which will be referred to as H. A receptor with IL-2 bound to it will be denoted by an asterisk. It is has been an issue of some uncertainty as to whether the α and β chains will form the H receptor complex in the absence of IL-2. However, recent work by Goldstein, et al.(1) has shown that these complexes are, in fact, formed, although the rate and equilibrium constants for this reaction have not yet been determined. These parameters are thus unknown and approximate values are used in the model.

In the presence of IL-2, H* receptor complexes may be formed by combination of α* with β or β* with α. These reactions are very rapid and the forward rate constants are assumed to be the diffusional rate constant limit for the surface reactions. The reverse reaction rate constants for these processes are not known, but are slow compared to the forward rate and will be taken as 1/100 of that value.

The chemical reactions for these processes are:

$$\alpha + \beta = H \; ; \quad K_H = [H] / [\alpha]\,[\beta] = k_H / k_{-H}$$

$$\alpha^* + \beta = H^* \; ; \quad K_{\alpha^*H} = [H^*] / [\alpha^*]\,[\beta] = k_{\alpha^*H} / k_{-\alpha^*H}$$

$$\alpha + \beta^* = H^* \; ; \quad K_{\beta^*H} = [H^*] / [\alpha]\,[\beta^*] = k_{\beta^*H} / k_{-\beta^*H}$$

4.2.2. IL-2 Receptor Binding

The equilibrium and rate constants for binding of IL-2 by the different IL-2 receptor species have been determined and are given in the Table 4.1. The chemical reactions are shown below.

$\alpha + [IL\text{-}2] = \alpha^*$; $K_{\alpha^*} = [\alpha^*] / [\alpha]\,[IL\text{-}2] = k_{\alpha^*} / k_{-\alpha^*}$

$\beta + [IL\text{-}2] = \beta^*$; $K_{\beta^*} = [\beta^*] / [\beta]\,[IL\text{-}2] = k_{\beta^*} / k_{-\beta^*}$

$H + [IL\text{-}2] = H^*$; $K_{H^*} = [H^*] / [H]\,[IL\text{-}2] = k_{H^*} / k_{-H^*}$

4.2.3. Receptor Internalization

The internalization rates for IL-2 receptor species have been determined in various studies (2, 3). The most interesting aspect of the internalization process is that when the beta chain or H receptor complex is bound to IL-2, they are internalized at a much faster rate than when IL-2 is not bound. The values are given in Table 4.1. The reactions are given below.

$$\left(\frac{dH}{dt}\right)_{\text{internalization}} = -k_{HI}\,[H]$$

$$\left(\frac{dH^*}{dt}\right)_{\text{internalization}} = -k_{H^*I}\,[H^*]$$

$$\left(\frac{d\alpha}{dt}\right)_{\text{internalization}} = -k_{\alpha}\,[\alpha]$$

$$\left(\frac{d\alpha^*}{dt}\right)_{internalization} = -k_{\alpha^*I}\,[\alpha^*]$$

$$\left(\frac{d\beta}{dt}\right)_{internalization} = -k_{\beta I}\,[\beta]$$

$$\left(\frac{d\beta^*}{dt}\right)_{internalization} = -k_{\beta^*I}\,[\beta]$$

4.2.4. Receptor Externalization

The IL-2 receptor externalization rates have not been measured directly but can be inferred from the steady state values of IL-2 receptors when the IL-2 concentration and internalization rates are known. The term externalization is here used to refer to the appearance of receptors from inside the cell. The source of these receptors may be either newly synthesized proteins or recycled molecules that had been internalized earlier. This model cannot distinguish between these sources so these phenomena are lumped into a single generation term that is assumed to be independent of surface receptor concentrations, but is not independent of the IL-2 concentration. As discussed in the first chapter, α chain synthesis and expression is stimulated by IL-2, whereas β chain synthesis levels are thought to be constant. This requires that the appropriate synthetic rates be explicitly entered and are assumed to be constant for the duration of the calculation. These rates are summarized in Table 4.1. The chemical rate equations for externalization are given below.

$$\left(\frac{d\alpha}{dt}\right)_{externalization} = k_{\alpha S}$$

$$\left(\frac{d\beta}{dt}\right)_{externalization} = k_{\beta S}$$

4.2.5. Receptor Balance Equations

The conservation of each species of receptor can now be written in terms of the processes that affect the receptor expression levels given above. These equations are:

$$\left(\frac{d\alpha}{dt}\right) = -k_{\alpha I}[\alpha] - k_{\alpha^*}[\alpha][IL\text{-}2] + k_{-\alpha^*}[\alpha^*] - k_H[\alpha][\beta] + k_{-H}[H] + k_{-\beta^* H}[H^*] - k_{\beta^* H}[\beta^*][\alpha] + k_{\alpha S}$$

$$\left(\frac{d\beta}{dt}\right) = -k_{\beta I}[\beta] - k_{\beta^*}[\beta][IL\text{-}2] + k_{-\beta^*}[\beta^*] - k_H[\alpha][\beta] + k_{-H}[H] + k_{-\alpha^* H}[H^*] - k_{\alpha^* H}[\alpha^*][\beta] + k_{\beta S}$$

$$\left(\frac{dH}{dt}\right) = k_H[\alpha][\beta] - k_{-H}[H] - k_{H^*}[H][IL\text{-}2] + k_{-H^*}[H^*] - k_{HI}[H]$$

$$\left(\frac{d\alpha^*}{dt}\right) = -k_{\alpha^* I}[\alpha^*] + k_{\alpha^*}[\alpha][IL\text{-}2] - k_{-\alpha^*}[\alpha^*] - k_{\alpha^* H}[\alpha^*][\beta] + k_{-\alpha^* H}[H^*]$$

$$\left(\frac{d\beta^*}{dt}\right) = -k_{\beta^* I}[\beta^*] + k_{\beta^*}[\beta][IL\text{-}2] - k_{-\beta^*}[\beta^*] - k_{\beta^* H}[\beta^*][\alpha] + k_{-\beta^* H}[H^*]$$

$$\left(\frac{dH^*}{dt}\right) = -kH^* I[H^*] + kH^*[H][IL\text{-}2] - k\text{-}H^*[H^*] - k\text{-}\alpha^* H[H^*] + k\alpha^* H[\alpha^*][\beta]$$

$$-k\text{-}\beta^{*}H\,[H^{*}] + k\beta^{*}H\,[\beta^{*}]\,[\alpha]$$

4.3. Model Program

The goal of the model is to predict the expression levels of each species of IL-2 receptor over time from a set of initial conditions, in other words, to solve the set of six simultaneous, non-linear differential equations given the receptor balances above. It was decided to use a finite difference analysis approach to this system, and the program was written in the Mathematica, v. 2.0 programming environment.

The application involved pseudo-linearization of the balance equations by constructing a 6 x 10 matrix where each row represented a single balance equation and each column represented all of the terms which could multiply any of the ten possible variables for the system. This matrix was then multiplied by the single 'step' size for the numerical system which, after analysis, was taken to be 1 second. The ten possible variables included all the receptor species; α, β, H, α^{*}, β^{*}, H^{*}, as well as the three non-linear terms that appear in the equations; $\alpha\,\beta$, $\alpha^{*}\,\beta$, and $\beta^{*}\,\alpha$. The final element of the vector was the constant 1, which is the relevant multiplier for the zero order externalization terms. The matrix of coefficients was then multiplied by the initialized variable vector, to generate a 6 x 1 dot product vector that contained the changes in the variables for the step. Four terms of zero were then appended to the dot product to generate a 10 x 1 vector which could be added to the initial variable vector, generating the changed values from one step for the first six terms. Finally, the three product terms of the variable vector were recalculated using the new values for the receptor species, and the process was repeated until 12 or 36 hours had been simulated.

Another feature of the model which should be explained is how the concentration of IL-2 is accounted for when IL-2 does not appear explicitly in the variable vector. The inherent rate constants, under saturating conditions, for binding and release of IL-2 from

the IL-2 receptors have been determined and are given in Table 4.1. To adjust for conditions where IL-2 was not saturating, these inherent rate constants were scaled by a Michaelis type expression:

$$k_{scaled} = k_{inherent} [IL\text{-}2] / (K_d + [IL\text{-}2])$$

where $k_{inherent}$ is the saturation rate constant and K_d is the dissociation constant for the equilibrium binding reaction between the receptor species and IL-2.

The code used for this application is presented as **Appendix B** to this thesis.

4.4. Results

4.4.1. Sensitivity Analysis of K_H Parameter

The parameters used in this model which do not arise from literature values are the kinetic constants for the association and dissociation reactions of the unbound receptors α, β, and H:

$$[\alpha] + [\beta] = [H]$$

The sensitivity of the model to these parameters was checked by running simulations of IL-2 receptor behavior for the case of saturating levels of soluble IL-2 when the value of the forward rate constant was changed over two orders of magnitude and the backward rate constant was fixed, thus altering the kinetic and equilibrium parameters. The results of 1 and 12 hour simulations are shown in Table 4.2. The 12 hour time points presented represent equilibrium values since the changes in the values from 11.5 hours to 12 hours are less than 2 parts per thousand for all of the receptor species. The equilibrium values

Table 4.2. Sensitivity of IL-2 Receptor Model to k_H Parameter

	$k_H = 2 \times 10^{-5}$		$k_H = 2 \times 10^{-6}$		$k_H = 2 \times 10^{-7}$	
Species	1 hr.**	12 hr.§	1 hr.*	12 hr.§	1 hr.**	12 hr.§
α chain	10,178	20,898	10,359	21,099	11,513	21,648
β chain	16	7.5	155	71	1299	641
H chain	200	190	196	184	184	170
α*	52	155	6	19	0.8	2.2
β*	0.1	0.05	0.3	0.09	1.8	0.5
H*	4282	3847	4320	3832	4261	3696

This table lists the values for IL-2 receptor species after simulations of 1 and 12 hours for the IL-2 receptor model where the forward rate constant k_H, defined by the reaction

$$[\alpha] + [\beta] \xrightarrow{k_H} [H] ;$$

is varied from 10^{-5} to 10^{-7} sec^{-1} $receptors^{-1}$. The case simulated is for saturating levels of IL-2.

**The 1 hour time points reflect transitional values between equilibrium and the initial guesses. The initial guesses for all three cases were; $\alpha_o = 5{,}000$; $\beta_o = 5{,}000$; $H_o = 100$; $\alpha^*_o = 50$; $\beta^*_o = 500$; and $H^*_o = 5{,}000$.

§The 12 hour time points have essentially converged to equilibrium values in all three cases.

generated by the model are independent of the initial guesses for the expression levels of the IL-2 receptor species.

As shown, the changes in α, H, α*, β*, and H* are not significant for changing k_H at the equilibrium points. The one significant difference, that of β chain expression, does not seriously effect the overall expression levels of IL-2 responsive receptors. For the 10^{-5} case, there are 4045 receptors expressed which can respond to IL-2 (β, β*, H, H*) while there are 4507 when the reaction constant is 2×10^{-7}, a difference of only 10%. If one just considers the number of signalling receptors present (β*, H*), the difference is even smaller at 3847 vs. 3696, a difference of only 4%.

The value of k_H used for the rest of the simulations presented, unless otherwise noted, is 2×10^{-6}. This value would present equal numbers of α, β, and H receptors when there are 1,000 receptors of each type present on the surface of a cell.

4.4.2. Model Predictions for Cases of Soluble IL-2

Simulations were performed where the IL-2 concentrations were presumed to differ from no IL-2 being present to low levels to high levels. The equilibrium values predicted by the model are shown in Table 4.3 and are independent of the initial guesses of the IL-2 receptor levels. The model converged to these equilibrium values after about 3 hours of simulation time.

The differences between the inputs for these simulations rest upon four constants: the externalization rate for the α chain, and the forward reaction rates for the binding of IL-2 to the α, β, and H receptor species. The reason for changing the constants for the IL-2 binding reactions are to simulate the amount of IL-2 available in the environment and is discussed in detail above. The change in the externalization rate for the α chain is to mimic the observed property of IL-2 to stimulate expression levels of the α chain. This property is observed in a variety of IL-2 sensitive cell types and is discussed in detail in Chapter 1.

Table 4.3. Equilibrium Values of IL-2 Receptor Expression for Different Levels of Soluble IL-2.§

Receptor Species	Presumed IL-2 Concentrations 0**	3.3pM∞	10 pM†	100pM†
α	783	3999	21,198	21,099
β	9202	1366	155	71
H	6584	4099	1813	184
α*	0	0.2	3.1	19
β*	0	0.3	0.05	0.09
H*	0	2546	3410	3832

§All of the trials converged to equilibrium values after less that 3 hours of simulated time.

**The $k_{\alpha S}$ value (externalization of α chain) for this concentration of IL-2 is assumed to be 1.4 sec^{-1}.

∞The $k_{\alpha S}$ value for this concentration is assumed to be 3.5 sec^{-1}.

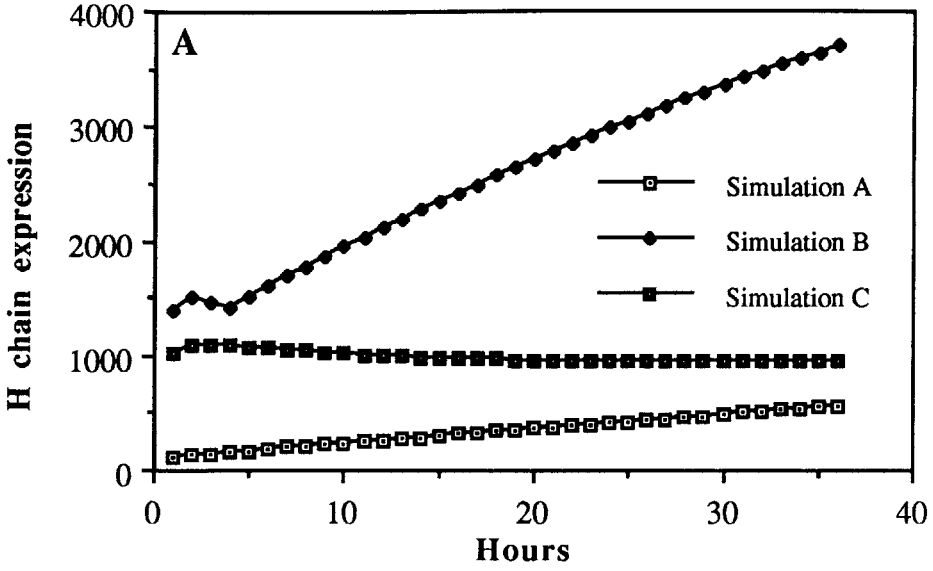
†The $k_{\alpha S}$ value for this concentration is assumed to be 7 sec^{-1}.

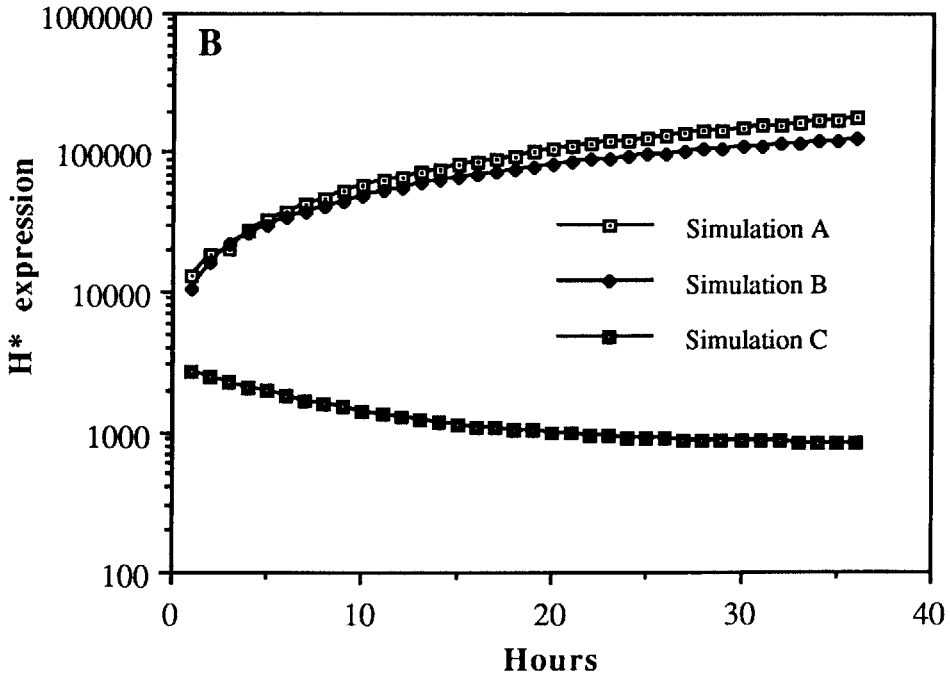
The most significant phenomena from these studies is that increasing IL-2 concentration drives down the expression of the free β chain to near zero levels and caused a decrease in the total amount of H receptor complex that was available. This down regulation of the H receptor complex has been previously noted (2, 3) and could explain the lower sensitivity of cells to IL-2 when they are pre-incubated in high IL-2 media that was observed in experiments from the preceeding chapter. The very small levels of β chain in the high IL-2 environment is also a confirmation of the model. The β chain was not detected by Scatchard analysis in cells which expressed the α chain and the H receptor complex. This simulation provides the explanation that kinetic and equilibrium conditions drive the number of free β chains to these low levels relative to the α and H receptors, thus the β chain would escape detection. The model also demonstrates that α chain expression increases significantly with increasing IL-2. Because the simulation assumes an increasing externalization rate of the α chain with increasing IL-2 concentration, this result is not surprising.

4.4.3. Model Predictions for the Case of Immobilized IL-2

For the case of cells incubating with immobilized IL-2, the model must be adapted to account for the inability of IL-2 bound receptors to be internalized and possibly for the finite amount of IL-2 which is available for binding.

Figure 4.1 illustrates the predicted levels of H, H*, and α chain expression under three different scenarios (the β, β*, and α* expression in these cases is small). For all three cases, it is assumed that IL-2 bound receptors cannot be internalized. In simulation A, there is assumed to be an infinite amount of IL-2 available for binding and the forward rate constants for the binding reactions are at their saturation levels. In simulation B, the forward rate constants for binding are adjusted to a level consistent with 10 pM of soluble


A
4000
3000
2000
1000
0
H chain expression
0
10
20
30
40
Hours
Simulation A
Simulation B
Simulation C


B
1000000
100000
10000
1000
100
H* expression
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10
20
30
40
Hours
Simulation A
Simulation B
Simulation C

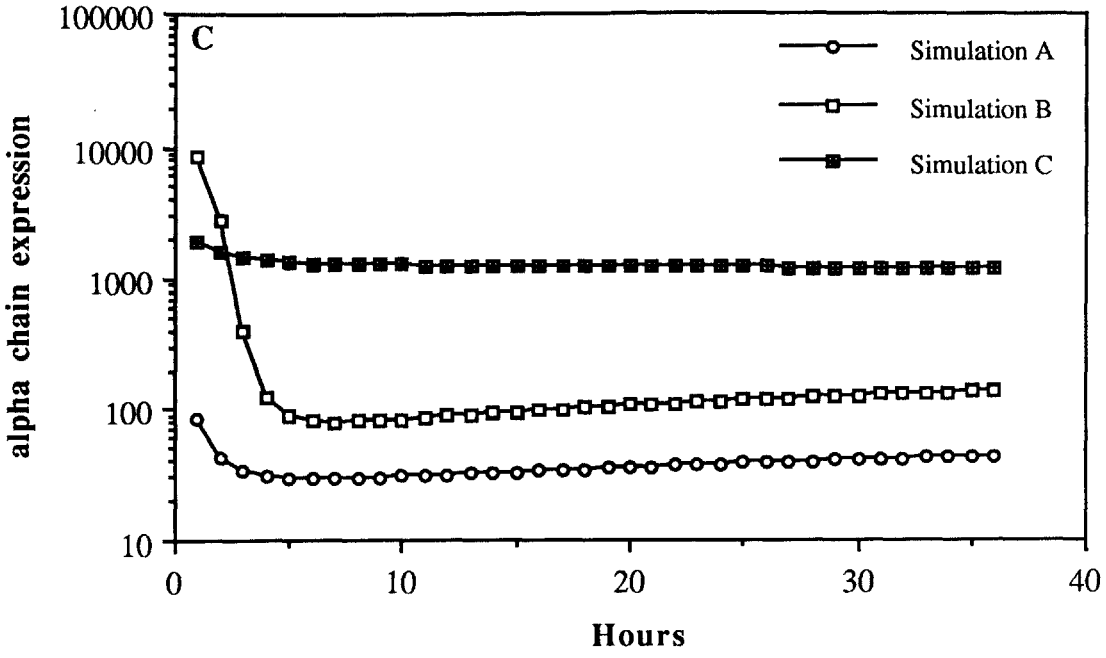

Figure 4.1. Simulated H and H* and α IL-2 Receptor Complex Expression Levels for Scenarios of Cells Incubating with Immobilized IL-2. Above are the predicted numbers of H (panel A) and H* (panel B) and α (panel C) chains for three different set of assumptions. In all cases it is assumed that no receptor bound to IL-2 can be internalized. In simulation A, it is assumed that there is an infinite amount of immobilized IL-2 available for binding. In simulation B, it is assumed that unbound IL-2 is available at low concentrations. In simulation C, it is assumed that there is no more IL-2 available for binding after a certain number of receptors have been bound, and that the externalization rates of new receptors is drastically reduced from the other cases.

IL-2, and for simulation C this value is adjusted down to 3.3 pM. For simulation C, it is also assumed that the externalization rates of the α and β chain are 0.4 sec^{-1} and 0.8 sec^{-1}, whereas for the other two cases these values are 1.4 sec^{-1} and 2 sec^{-1} respectively. These parameters are listed in Table 4.4. As shown in Figure 4.1, the assumptions about IL-2 receptor behavior with immobilized IL-2 cause the predictions of receptor level expression to differ drastically from the soluble case.

By assuming that bound IL-2 receptors cannot be internalized, the major sink of receptors from the surface is removed. This change has two dramatic effects on the model predictions. The first is that the immobilized IL-2 simulations do not reach steady state even after 36 hours, whereas the soluble simulations approached steady state after only 2 or 3 hours. The second is that the immobilized IL-2 simulations A and B, which presume that IL-2 is available for binding, show an ever increasing level of receptor expression for the H chain. The reason for these changes is that without the internalization of bound receptors, the only way the simulations will reach an equilibrium point is when the externalization rates equal the slow internalization rates of unbound receptors. Since the affinity constants of the receptors for IL-2 are relatively strong, the only ways there will be sufficient numbers of unbound receptors to be internalized are when there is no more IL-2 to bind, as in simulation C, or when the number of bound receptors becomes huge so that the dissociation rate between IL-2 and the receptors becomes significant. Thus the large number of receptors projected in simulations A and B, and the lack of a steady state until this large receptor buildup is present.

4.5. Discussion

IL-2 mediates its effects by binding to specific IL-2 receptors that are expressed on the surface of sensitive cells. These receptors have three distinct forms that bind to IL-2 with differing affinities and are produced by the cell at different rates. The low affinity

receptor (α chain) binds to IL-2 with a dissociation constant of 30-100 nM. The intermediate affinity IL-2 receptor (β chain) binds to IL-2 with a dissociation constant of about 10 nM and consists of at least two proteins. These two receptors can combine to form a third IL-2 binding moiety called the high affinity receptor (H chain) which has a dissociation constant of 30 pM.

A mathematical model was constructed which incorporated equilibrium and kinetic data of the IL-2 receptor system. The model was used to predict receptor expression levels of all of the different forms of the IL-2 receptor for soluble IL-2 systems, and these predictions were checked by comparison with the control studies in previously discussed experiments. The model was also applied to immobilized IL-2 incubations and the parameters which govern the model were modified to force the predictions to follow the observed trend. These changed parameters offer some insights into the behavior of cell incubated with immobilized IL-2.

The basis of the mathematical model was a collection of rate equations which govern the appearance and disappearance of IL-2 receptor species on the cell surface. The processes which effect expression levels were organized into four groups: internalization, externalization, IL-2 association and dissociation, and receptor combination and separation. Internalization is the process where receptors are removed from the cell surface and is the ultimate sink for the receptors. Externalization refers to the process by which receptors move from inside the cell to the cell surface and includes the synthesis of new receptors and the recycling of receptors which may have been previously internalized, processes which are lumped into one parameter for this model and is the ultimate source term for all receptors. IL-2 association and dissociation refers to the binding and dissociation of IL-2 by receptor species; and receptor combination and separation refers to the reactions by which the α and β chains can combine to form the H chain, and similar reactions involving species bound to IL-2. These last two processes do not change the total number of receptors on the cell surface but alter the species of receptors that are present.

The dynamics of the model are driven by movement toward equilibrium conditions between competing receptor species and IL-2, and by two decisions that are made by the cells, the externalization and internalization rates for the receptors. This model uses fixed values for the duration of its simulation, the only adjustment made to changing environmental conditions is the externalization rates of the α and β receptors, which have been observed to be responsive to the presence of IL-2. The model is not really designed to predict the behavior of IL-2 responsive cells. It is instead designed to simulate the equilibrium points for receptor level expression that are projected by considering simple physical and chemical rate processes. The degree to which cellular behavior can be correlated to model predictions is then the degree to which these elementary processes drive the cellular response. When the model fails, it means that other decisions are being made by the cell which defy the assumption of constant values made by the model.

For the case of soluble IL-2, the model does a good job predicting the down regulation of high affinity receptor chains and the increase in total α chains with increasing IL-2 concentrations for two reasons. The first is that the receptor association rates, the IL-2 binding rates, and the receptor internalization and externalization rates were all defined from experiments which used soluble IL-2. There is thus a high confidence level that the rate processes are adequately valued for the interactions between cells and soluble IL-2. These simple physical and chemical rate processes are also apparently sufficient to achieve the desired changes a cell needs to make to adjust to changing levels of soluble IL-2. The second reason is that one of the decisions made by the cell, the externalization rate of α chain receptors, was adjusted upward with increasing amounts of IL-2. This phenomenon has been observed in experiments, but this adjustment nonetheless is an input which really drives the changes in receptor level expression. The only other changes made in the model in response to changing IL-2 concentration is altering the forward rate constants for receptor - IL-2 binding, a change which is firmly based on chemical principles.

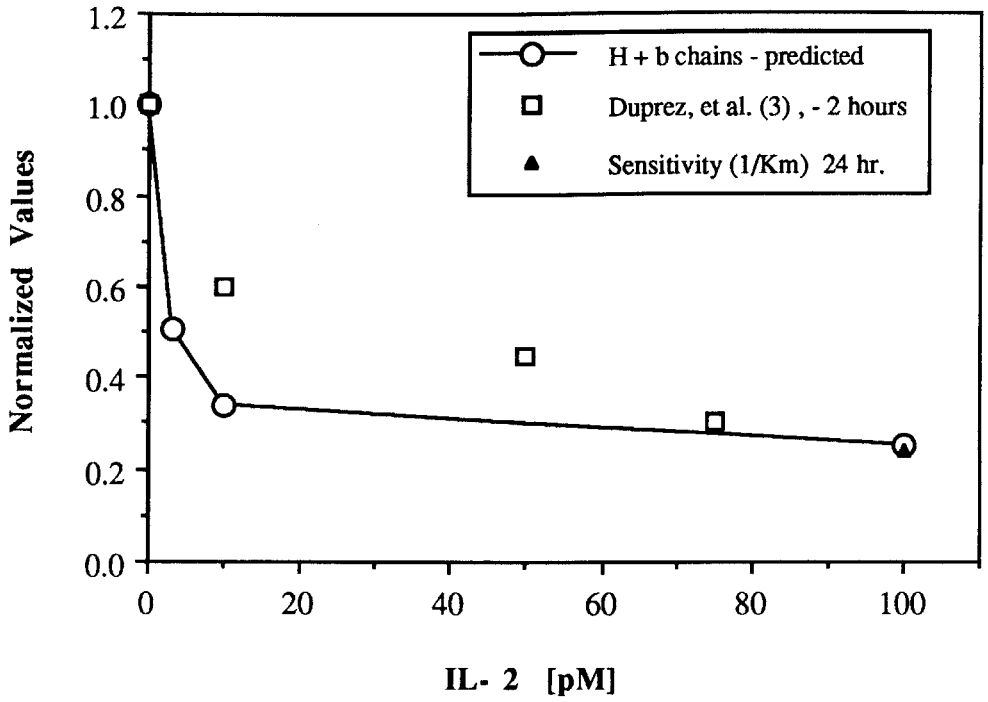

Figure 4.2. Normalized Values of Receptor Expression Level (Predicted) and Sensitivity to IL-2 (Experimental). The model predictions of IL-2 high (H) and intermediate (b) affinity receptors are shown along with data displaying the change in sensitivity to IL-2 after pre-incubation with IL-2. Sensitivities are 1/Km values shown in table 3.1. Data portrayed is normalized by its highest value.

As shown in Table 4.3 and in Figure 4.2, the model can predict the trend of IL-2 receptor level expression for cases of cells incubating with soluble IL-2. The experimental observations for this system are that there is little or no detectable β chain for cells with soluble IL-2, α chain levels increase dramatically, and that H chain expression decreases. Figure 4.2 shows how the model predictions for H and β chain expression compare to experimental data collected by Duprez (3) on intermediate and high affinity receptor expression levels. The model does a satisfactory job predicting the trend of the data and the values for high IL-2 levels (it predicts reasonably well the altered K_m observed in earlier studies), but not for points at lower IL-2 concentrations. The model could be made to fit the data very well in this region by altering the assumed externalization rate of the α chain. There is no hard data available for this parameter and if lower rates were assumed, the predicted values would be higher at the 3.3 and 10 pM points. Again, this illustrates the weakness of the model to account for regulatory decisions of the cell. At very low IL-2 levels and at high IL-2 levels the externalization rate of the α chain is close to constant, but in the transition regions where this rate rapidly changes, the model will suffer. A future improvement to this model would be to incorporate an explicit IL-2 sensitivity into the parameters.

For the case of immobilized IL-2, several difficulties arise. The first is that a new physical situation is encountered where the IL-2 receptors are meeting immobilized IL-2 on a surface instead of soluble IL-2 in a volume. In addition, the rate constants used for the IL-2 binding reactions are taken from a literature which evaluated these reactions with soluble IL-2; and finally, the internalization rate of IL-2 bound receptors must be set at zero. The behavior for immobilized IL-2 is simulated by the model under three different conditions that incorporate increasing numbers of new assumptions. These are presented in Figures 4.1 and 4.3 and the parameters are summarized in Table 4.4.

In simulation A, the model was altered from the soluble case only by making the assumption that all IL-2 bound receptors could no longer be internalized. It was also assumed that there was a surplus of IL-2 available to be bound and that the binding kinetics were identical with the soluble case. The result, as shown in Figures 4.1 and 4.3, was that the assumptions removed the major sink from the chemical equilibrium, the rapid internalization of IL-2 bound receptors, without removing the source terms. Consequently, receptors just keep piling onto the cell surface, moving toward an equilibrium point where externalization is equal to the internalization of unbound receptors. This level is not reached within 36 hours but the total numbers of receptors are already far higher than those observed in our experiments and presented in Chapter 3.

The assumptions made to correct this disparity were that the amount of IL-2 available for binding is not unlimited but is, rather, small. This assumption was realized by adjusting the forward IL-2 binding constants of the receptors down by an order of magnitude and this case is presented as simulation B. As seen in Figure 4.3, there was a slight decrease in predicted total receptor expression. Since fewer receptors were binding to IL-2, more were available to be internalized. Yet the source terms still overpowered the available sinks and the simulation predicted that receptor levels would still far exceed those found experimentally.

The final set of predictions, simulation C, addressed the disparity between source and sink terms by arbitrarily lowering the values of the externalization rates for the α and β chains. In addition, the forward binding reaction constants were further reduced, rendering the situation analogous to one where most of the IL-2 on the membrane has already been bound by receptors and little is left available. As shown in Figures 4.1 and 4.3, these changes tilt the balance between source and sink terms and the number of receptors actually declines as few new receptors are externalized, and few can bind to IL-2 so that the remainder can be internalized.

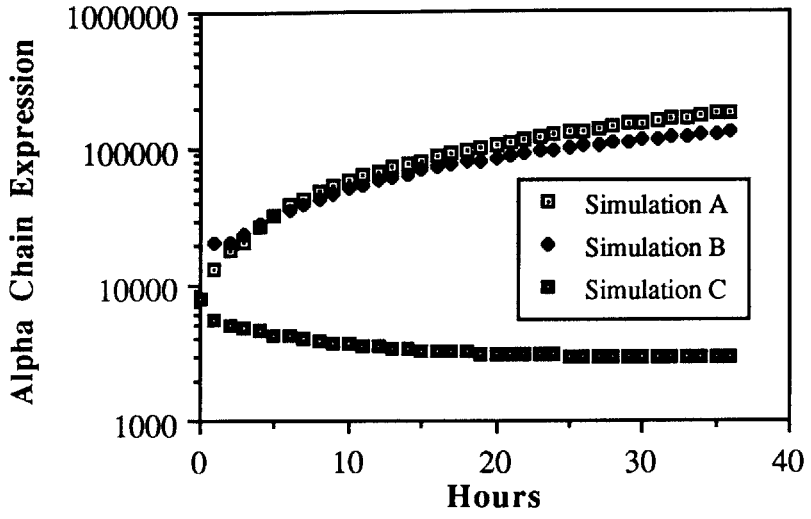

Figure 4.3. Total Predicted α Chain Expression in Immobilized IL-2 Simulations. The figure displays the total number of receptors which contain an α chain (= H + H* + α + α*) from the three immobilized IL-2 simulations.

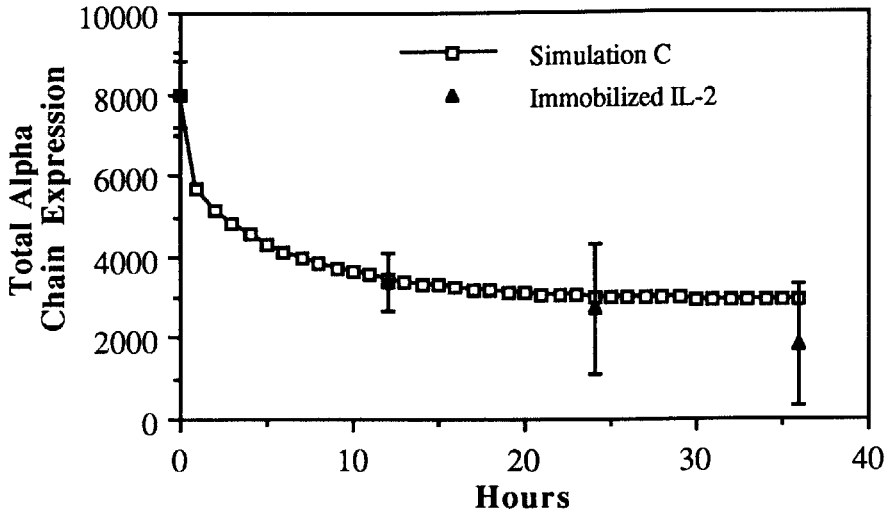

Figure 4.4. Expression of Total α Chain Predicted by Simulation C and from Anti-IL-2R α Staining Experiments. The trend for total IL-2R α chain expression (= H + H* + α + α*) found from fluorescently labeled antibody staining experiments is shown along with the predicted values of IL-2R α from simulation C.

Figure 4.4 shows how the predicted values of receptors using simulation C compares to the experimental data presented in Chapter 3 on the number of IL-2 α chain present for cells incubated with immobilized IL-2. The experimental data gave relative fluorescent values for cells incubating on the immobilized IL-2 membrane. For the sake of comparison, the zero time point value was arbitrarily set to equal 8,000, consistent with reported values (5). Clearly, by adjusting the parameters in the manner described, it is possible to mimic the trend found experimentally.

The results of these simulations demonstrate that to predict the data observed experimentally, it is necessary to incorporate further assumptions into the model or to directly measure certain parameters for future work. The assumption that there is a finite amount of IL-2 available for binding by cells in the immobilized IL-2 incubations is obvious and justifiable since there is a finite amount of IL-2 bound to the immobilized IL-2 matrix. However, the studies included in Chapter 2 show that the calculated amount of IL-2 available for each cell is 77,000 molecules, far in excess of the number of receptors on a typical CTLL-2 cell, which has been found to range from 10,000 to 30,000 (5). The figure of 77,000 molecules refers to IL-2 molecules bound to the matrix, but does not consider the chemical or steric processes that may render the bound molecules inactive. This modelling allows an order of magnitude approximation for these deactivating effects, predicting that only about 10% of the total bound molecules are active.

Another interesting feature predicted by the model is the shutdown of receptor externalization. These externalization levels are even less than those presumed for the case of no IL-2. This might indicate that once cells achieve a certain number of receptors bound to IL-2, further externalization is turned off. Applying this principle to the case of soluble IL-2, it would seem that high levels of IL-2 should actually reduce the total number of receptors over time. This phenomenon was observed in the K_m value and immuno-fluorescent studies on CTLL-2 cells incubated with 1 ng/ml IL-2 and presented in Chapter

3. This phenomenon, however, would only become apparent after the initial response of cells to the soluble IL-2, which is increased externalization of IL-2 receptors.

A speculation based on this phenomenon suggests that the immobilized IL-2 membrane, in saturating some critical amount of IL-2 receptors, renders its effects by making the CTLL-2 cells IL-2 independent. Thus the cells neither grow, nor die, nor express IL-2 mediated functions. This state could not be reached by using soluble IL-2 because the cells, continually internalizing IL-2 bound receptors and digesting IL-2, would not remain in an IL-2 sated state.

The conclusions from applying this mathematical model of IL-2 receptor expression to the simulation of CTLL-2 cells with soluble and immobilized IL-2 is that such a model can be constructed from values in the literature, and that it can be used to predict certain behavior of cells incubating with soluble IL-2. The model shows that with just one adjustable parameter, the externalization rate of the α chain, equilibrium and kinetic properties of the IL-2 receptor system alone are adequate to predict IL-2 receptor expression levels. The model has also proved useful as a tool to probe the interaction between immobilized IL-2 and CTLL-2 cells.

To improve the performance and utility of the model, it would be very useful to perform a study which characterizes the externalization rates of the IL-2 receptors as a function soluble IL-2 concentration. With this data, the model could more accurately predict IL-2 receptor level expression of sensitive cells with soluble IL-2 and become a useful tool for understanding some interesting aspects of cell behavior uncovered in Chapter 3. One such application might be to interpret the observation that CTLL-2 cells are apparently recruited to enter a proliferative cycle when incubated with soluble IL-2, where the probability of this event occuring is proportional to the soluble IL-2 concentration. Using the refined model, the growth data presented in Chapter 2, and the population distribution statistics that are available from the fluorescent staining data in Chapter 3, it should be possible to define the probability curve for a cell to enter a proliferative cycle in

terms of the number of receptors expresseed and the amount of IL-2 bound. This information would be very useful for the design of control schemes to optimize IL-2 presentation in culture when the goals are IL-2 driven cell proliferation or stasis.

Other improvements of the model would include incorporating the IL-2 concentration explicitly into the model equations. This could be done for the soluble case by introducing subroutines which would calculate the forward rate constants for the IL-2 binding reactions using the scaling parameters discussed earlier, and it could be done for the immobilized IL-2 case by not only having a recursive loop to recalculate the binding rate constants but also by recalculating the amount of free IL-2 available to be bound. With these additional data, the model could serve as an even more effective tool to distinguish what IL-2 receptor behavior is a consequence of regulatory decisions made the cell, and what is driven by the equilibrium and rate processes of the system.

4.6. References

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APPENDIX A: IL-2 IN THERAPY

A.1. Introduction

As a potent stimulator of immune cell growth and function, IL-2 is an obvious candidate for use as a therapeutic agent in pathologies that can be addressed by the immune system. From conditions such as leprosy (1, 2), to malaria (3), to rheumatoid arthritis (4) and virally infected cells (5, 6), IL-2 could have a role as therapeutic agent. Yet the application of IL-2 in cancer therapy is by far the best studied use of the lymphokine. This is because of both the magnitude and ubiquity of the disease and because IL-2 has been shown to have a tantalizing activity; culturing peripheral blood lymphocytes (PBL) or large granular lymphocytes (LGL) in high titers of IL-2 generates cells that can selectively lyse tumor targets *in vitro* (7, 8). After many studies, some of which are described below, IL-2 has been approved by the Food and Drug Administration of the United States for use in the treatment of renal cell carcinoma as of May of 1992. Thus IL-2 is currently available as a prescription drug.

Because of its role as an immune regulator, the use of IL-2 in cancer therapy falls under the rubric of "immuno-therapy". Immunotherapies can generally be characterized by two different approaches. "Active" immunotherapies involve the use of agents to invigorate patients' immune systems and that, hopefully, will lead to tumor regression. This approach would include procedures like the perfusion of IL-2 into patients, or the use of Bacillus Calmette-Guerin (BCG), Corynebacterium-parvum, levamisole, and other immunogenic agents (9). Another approach is "passive" or "adoptive" immunotherapy in which the patient receives reagents such as antibodies or reactive cells that already possess antitumor reactivity (10, 11). Combinations of these approaches are, of course, also possible. IL-2 has been used in all three modes, it has been perfused directly into patients,

it has been used *ex vivo* to activate tumoricidal cells, and the preceding methods have been used in combination.

A.2. LAK Cell Generation and Characterization

The most famous of the combinational IL-2 based approaches was the use of lymphokine activated killer cells (LAK). LAK cells are defined as cells that are generated from lymphokine rich culture, usually IL-2, and which can specifically lyse fresh tumor target cells *in vitro*, though not fresh normal cells (12). The most striking feature of this phenomenon is the ability of these cells to lyse tumor cell preparations that are natural killer cell resistant, either syngeneic or allogeneic, primary or metastatic (7). This feature distinguishes LAK cells from natural killer cells which are greatly limited in their ability to lyse fresh tumor targets.

Since LAK cells are defined by their functional activity, much work has gone into determining what types of cells are responsible for the phenomenon. The approach for these studies is to selectively delete populations of cells by complement lysis using monoclonal antibodies against specific phenotypic markers, or to sort and analyze the cells using the phenotypic markers and a fluorescent-activated cell sorter (FACS). Using these methods on the precursor population PBL, the cells which become LAK have been variously characterized as OKT-3$^-$, Leu-1$^-$ null cells (13); CD3$^-$, Leu-19$^+$ NK cells (14); Lyt-2$^-$, L3T4$^-$, asialoGM$_1^+$ cells (15); CD3$^-$, CDw16$^+$, NKH1(=Leu-19)$^+$ multiplicity of precursors (16); Lyt-2$^-$, L3T4$^-$ not NK cells (17); Lyt-2$^-$, aGM$_1^+$, NKH1$^+$ NK-like cells (18); or DM-1+ cells (19). A report which focused on the morphology of the LAK precursor cells determined that the large agranular lymphocyte fraction of nylon wool passed lymphocytes could be induced by high titers of IL-2 to expand and become large granular lymphocytes, which then acquired LAK activity (20).

Some of the differences in the above phenotypic studies may be accounted for by differences in procedure and in the definition of LAK activity. The studies were not consistent in the amount of time cells were cultured in IL-2, or in the amount of IL-2 used. Yet, the data from these studies is surprisingly consistent. It is agreed that the precursor population are not CD8 (T8) cytotoxic/suppressor T cells nor are they CD4 (T4, L3T4) helper/inducer T cells. The cells are also not B cells, so the classically antigen driven lymphocyte population has been ruled out. The population remaining consist of "null" T cells, so named because the cells express some T cell markers but do not express the T cell receptor (these phenotypes were named before the discovery of the γ/δ T cell receptor complex). The only controversy then, is whether these precursor cells are NK cells or not. Some of the studies claimed that the precursors were NK cells, since lysing cells that express the NKH1 (leu-19) natural killer cell marker would delete the LAK activity. In other studies it was shown that if one fractionates the cells according to density, the LAK precursors exist in a fraction that has only a very small percentage of the NK activity of the total population. So the controversy is dependent only on whether NK cells are defined by phenotypic marker or by effector function. The consensus would be that the LAK precursors were cells of the NK lineage that did not express full NK function.

The phenotype of the LAK effector cell population has also been characterized. Published studies have reported that the effector cells are: OKT-4$^-$, OKM-1$^-$, OKT-3$^+$(=CD3), Leu-1$^+$ CTL like cells (13); CD3$^-$, Leu-19$^+$ NK cells (14); CD3$^-$, NKH1$^+$, CD8$^-$, CDw16$^+$ cells (16); aGM1$^+$, NKH1$^+$, Lyt-2$^+$ cells (18); CD3$^-$, CD2$^+$, CD8$^-$, CD7$^+$, T11$^+$ cells (21); ; and CD8$^+$, B220$^+$, Ly-24$^+$, NKH1$^-$, or NKH1$^+$, CD8$^-$, B220$^+$, Ly-24$^+$ cells (22). Once again, the procedures used to generate LAK cells were not uniform in all of the studies, and importantly, not all of the studies performed the necessary control of examining the cytotoxic activity of the activated killers against fresh normal cells.

These factors go far to explain the heterogeneity of the results. The data on the LAK-effector cell population really point toward two different kinds of cytolytic cells. The first

are the NK like cells that are NKH1 positive and T3 (=CD3) negative. The second are cytotoxic T lymphocyte cells being CD3 and CD8 positive and NKH1 negative. Since the LAK precursor population is CD3 negative, this second active phenotype might have arisen in 2 ways: a CD3-CD8 negative cell type differentiated into a CD3 positive type in IL-2 culture, or that CD3-CD8 positive cells were stimulated and expanded in the IL-2 culture and had some lytic activity against the indicator cell lines. The second explanation is the most reasonable since the lytic activity shown by the CD8 cells was not shown to be specific for tumor tissue and since the tumor targets were allogeneic cultured cell lines. This explanation is even more likely when it is considered not all of the studies reported LAK activity among the CD3 positives. Thus the LAK effector cell is likely to be of NK lineage although other cell types may also contribute to tumor lysis (23).

Two other reports in the literature characterize the phenotypes of more specialized activated killer cells: one deals with plastic adherent LAK cell populations (24) and the other with activated killer cells expanded in a novel manner (25). The results of these studies are consistent with the conclusion that the effectors are NK like cells.

The supposition that the LAK effector cells are kinds of natural killer cells is also supported by studies which show the involvement of other lymphokines in the development of LAK activity. Culturing peripheral blood lymphocytes in high titers of IL-2 leads to production of γ-IFN by the cultured cells (26). The subsequent development of LAK activity has been shown to be dependent on the presence of the γ-IFN (27-29), if one blocks the γ-IFN with neutralizing antibody, one also blocks the development of the LAK activity. Moreover, one can generate LAK activity just by treating PBL with exogenous γ-IFN or α-IFN, but if one adds anti-IL-2 neutralizing antibody to these cultures, the activity is also ablated (30). The conclusion that can be derived from these reports is that both γ-IFN and IL-2 are required for the development of LAK activity, and that exogenous addition of one of the lymphokines stimulates secretion of the other (although the addition

of IL-2 is more stimulatory for LAK than the addition of γ-IFN). γ-IFN is a potent activation factor for NK cells.

A.3. LAK Cell Regulation

LAK cell activity is very sensitive to the culture conditions and the population of cells present. Cultures must be constantly refreshed to keep the effector cell population expanding (31, 32) and the presence of differing cell types can affect the generation of activity (33). In addition, other lymphokines besides IL-2 and γ-IFN can affect LAK cell generation. IL-4 is capable of stimulating activated killing from splenocytes (34) or PBL (35) as well as inhibiting the IL-2 induced response (36-39); and IL-7 (40, 41) and TNF (42, 43) both augment the development of LAK activity.

LAK cell generation can be inhibited in culture by the addition of phorbol ester (44) and by IL-1 receptor antagonist (45). In addition, other cell types can affect LAK activity. Polymorphonuclear neutrophils suppressed the induction of LAK cells in a dose dependent manner when they were present in the IL-2 culture (46) and T cells were able to suppress the response of a non-adherent subpopulation of LAK cells (47). Natural killer cells also seem able to suppress LAK activity. It has been shown that NK cells activated by IL-2 or a viral infection are responsible for the rejection of LAK cells in mice (48). This has been confirmed in an *in vitro* study which demonstrated, in effect, that LAK cells can kill LAK cells: human LAK cells that were expanded in IL-2 rich culture for 4 days were capable of lysing LAK cells from the same donor that had been expanded in culture for 8 days, and vice versa (49). The results from this study should not be over-interpreted, but it does illustrate that the cell products of the IL-2 expansion can themselves elicit a strong immune reaction.

In order to understand why different lymphokines or cell types affect LAK activity as they do, more has to be known about the induction and killing mechanisms of LAK cells.

Only a few glimpses are now available on this subject. γ-IFN is not only able to augment LAK activity in culture, it is also able to protect target cells from NK(50, 51) and LAK cell lysis (52). As little as 1-10 U/ml of γ-IFN present for 3 hours can mediate this response and it will last from 48-72 hours after the γ-IFN has been removed. The binding between the LAK cells and their targets is not effected (53), so the mechanistic explanation must be either that γ-IFN makes target cells less susceptible to the lytic action of the LAK cells, or that the γ-IFN reduces the expression of epitopes that trigger LAK cell action. Another study (54) has shown that treating tumor targets of LAK cells with trypsin reduced the killing of these cells by about 50%. This suggests that LAK cells specifically recognize trypsin-sensitive molecules on the tumor cell surface. This was also demonstrated by extracting tumor cell membranes and reincorporation of the membranes around cell sized hydrophobic beads. These beads were able to competitively inhibit the lysis of untreated tumor cells, and if the beads were treated with trypsin, the inhibition was ablated. It was noted that membranes from one kind of target cell could block the lysis of another.

A.4. LAK Cell Therapy

The *in vitro* ability of LAK cells to specifically lyse fresh tumor samples has led to the investigation of the phenomenon *in vivo*, both in animal models and in human clinical trials. The original demonstrations of LAK activity *in vivo* came from Rosenberg and colleagues in a mouse model where the animal was injected with tumor cells from a methyl-cholanthracene induced sarcoma and therapy was initiated 3 to 10 days later (55, 56). By this time, tumor nodules are established in the lung and are invading from the pulmonary capillaries in alveoli. The basic protocol was to perfuse 10^8 LAK cells into the animals once or twice and to inject i.p. a large bolus of IL-2 3 times a day for up to 10 days after the initial perfusion of LAK cells. This protocol came about from the observation that perfusion of LAK cells alone, or injection of IL-2 alone, were not very effective in

reducing the tumor in the animals, but the combination was effective. Applications of this basic protocol were also shown to be effective using hepatic tumors (57, 58), brain tumors (60), and squamous cell carcinoma modes (61).

This protocol was also investigated in human clinical trials (62, 63). Patients received both autologous LAK cells obtained from multiple leukapheresis and up to 90 doses of IL-2. Out of 41 patients with a variety of tumors, 14 objective responses were seen as defined by at least a 50% reduction in the volume of all measurable tumor. These responders included 5 out of 10 patients with melanoma, 5 / 5 patients with renal cell carcinoma, and 3 of 14 patients with colorectal tumors. The response for other tumor types was not impressive: 0 / 5 for sarcoma, 1 / 4 lung adenocarcinoma, and no response from individual cases of lymphoma, gastrinoma, and esophageal carcinoma. There were significant toxicities associated with this protocol that included acute flu symptoms and life threatening pulmonary edema. These symptoms could be attributed to the infusion of IL-2.

The bolus injections of IL-2 that were given to patients in ranged from 10^5-10^6 U/kg. Another protocol (64) was devised that used a continuous perfusion of IL-2 in amounts up to $5x10^6$ U/m^3/day. It was hoped that the toxicities elicited by the bolus injection of IL-2 would be reduced. The procedure required patients to be given LAK cells and then be perfused for 5 days, followed by 5 days of rest and leukapheresis for the next cycle. For this study, of the 40 patients evaluated there were 13 partial responders and 2 minor responders. 5 / 10 patients with melanoma responded, 3 / 6 with renal cell carcinoma, 1 / 5 with lung cancer, 1 / 1 with ovarian cancer, 0 / 13 with colon cancer and 0 / 1 with breast cancer. Once again, there were serious toxicities associated with the procedure. All patients suffered from fatigue, anemia, fever, eosinophilia, and rashes. Other toxic effects were also manifest.

One study directly compared the use of bolus injection every eight hours of IL-2 vs. continuous infusion of the lymphokine as the method accompanying perfusion of LAK cells (65). It was found that the continuous perfusion method yielded greater

immunomodulatory changes such as higher levels of IL-2R$^+$ and Leu-19$^+$ circulating lymphocytes, and higher levels of LAK cells from leukapheresis. The continuous infusion method also yielded greater lymphocytosis. The toxicity levels of the protocols were judged to be similar and the only 2 responding patients of the 24 tested were in the continuous infusion group. A separate study using sheep (66), however, determined that while both methods caused cardiopulmonary toxicity, but that pulmonary hypertension, systemic hypotension, and gas exchange were worse in the continuous infusion group. The conclusion is that continuous infusion of IL-2 seems to be more effective clinically than a bolus injection as methods to administer adjunct IL-2 in LAK cell therapy, but it is also associated with greater toxicity.

The results of these clinical trials raised several critical points. The results were promising for the treatment of renal cell carcinoma and melanoma, but disappointing for all other types of disease. The procedures also involved serious side effects and were difficult to tolerate, and the investment of material and labor were significant. Each leukapheresis required 30 to 40 separate roller bottles, each containing a liter of media plus 10^6 U of IL-2 (63). It became clear that improvements in the toxicity , the cost, and the efficacy of the therapy would have to emerge before LAK cells administration could become a widespread tool in oncology (67).

A.5. Tumor Infiltrating Lymphocytes

Natural killer cells are not the only cell types in the immune system repertoire that are capable of lysing tumor cells. When solid tumors are excised and cultured in IL-2, a population of T lymphocytes that had infiltrated the tumor will expand in culture (68, 69). These cells are known as tumor infiltrating lymphocytes or TILs and can also present potent antitumor activities.

The existence of lymphocytes infiltrating into solid tumors has been known for many decades (70, 71) and a large degree of infiltration has generally been associated with a more favorable prognosis for the patient (72-75). Yet early studies of TILs did not demonstrate any significant antitumor or natural killer activities among these cells (76-80). A few studies demonstrated that TIL from colorectal tumors have antitumor activity against autologous tumors, but not against the cell line K562, a standard NK cell target (81-83). However, with the discovery of effects of IL-2 and LAK cells, TILs were reevaluated as antitumor effectors and it was determined that by expanding TIL in IL-2, significant tumoricidal activities could be generated (69, 84, 85). Antitumor TIL effector cells have now been generated from a wide variety of solid tumors including lung (86-88), liver (89), melanomas (69, 90), kidney (91, 92), squamous cell carcinomas of the head and neck (93), gliomas (94), and colorectal tumors (95).

Preparation of TILs is generally accomplished by obtaining surgical sections of tumor, removing necrotic and connective tissue, and then mincing the remaining the remaining section and forcing it through a mesh into media. The fragments are incubated with hyaluronidase and collagenase for up to 3 hours and then centrifuged and washed. These cells are spun through a density gradient to remove dead cells, erythrocytes, and other non-lymphocytes. A second centrifugation with discontinuous gradients of Ficoll-Hipaque could separate the tumor cells from the lymphocyte population. The TILs are expanded by culturing in supplemented RPMI 1640 media with 1,000 U/ml of IL-2 added. The time required to generate working numbers of TILs varies from study to study, but it generally seems that 5 - 8 weeks are needed to generate 10^8-10^9 TILs from original tumor samples of 0.5 - 3.0 grams. However, it appears that only about 75% of solid tumor samples yield vigorous TIL populations. In addition, TILs from lesions that were thought to be metastatic either do not proliferate or were significantly delayed in their proliferative responses (89, 93).

TILs have been extensively characterized according to their phenotype (91-95). These cells appear to consist of at least 90% mature T cells that express the pan T cell marker CD3. Less than 2% of the cells seem to be of the NK type. The reports also demonstrated that it is these CD3 positive populations that mediate the antitumor activities of TIL and that these cells are distinct from the NK like effectors in LAK cell cultures. Confirming evidence for this fact is that TILs are MHC restricted and do not lyse a broad range of tumor targets, although they may be active against some allogeneic tumor lines. These T cell effector populations can be subdivided into $CD8^+$ and $CD4^+$ cells, with the majority (60%-80%) expressing the $CD8^+$ cytolytic/suppressor phenotype and the rest CD4 helper/inducer cells. The $CD8^+$ cells apparently have the greatest proliferative potential in the first few days of culture, but the $CD4^+$ cells will eventually predominate in longer term culture of over 60 days (92, 96, 97). Some of the kinetics of tumor lysis by the T cells have also been established. The $CD8^+$ and $CD4^+$ subpopulations do not demonstrate tumoricidal activity in 4 hour Cr^{51} release assays, yet both subsets can effectively lyse tumor over a 72 hour assay period. This is in contrast to NK cells which show significant activity in the 4 hour assay and it demonstrates tumoricidal effector activity in the $CD4^+$ subset (91). The expansion of TIL in culture will falter after several weeks without restimulation with either the tumor antigen or lectin (88-92, 96); periodic restimulation has maintained the tumoricidal activities of the culture for more than 6 months (88).

In vivo studies using TIL in a murine model system have shown that TILs are 50 to 100 times more potent in mediating the regression of established tumor than LAK cells (98, 99). Successful therapy with TIL is also dependent upon simultaneous administration of IL-2 as well as the pretreatment of the tumor bearing host with cyclophosphamide or with total body irradiation. In human clinical trials, a phase I study showed that cyclophosphamide, IL-2 and TILs can be given safely in combination therapy (100). In a study on 20 patients with malignant melanoma (101), the combined IL-2, cyclophosphamide, and TIL treatment produced objective responses in 9 of 15 patients

who had not had prior IL-2 therapy and in 2 of 5 patients who did have prior IL-2 treatment. The length of regression lasted from 2 to more than 13 months. The toxic side effects of IL-2 were present in all patients, but the treatment course was a relatively short 5 days and the toxic effects were reversible. TILs have also been used in the first human trials with transfected cells (102). TILs from 5 patients suffering from malignant melanoma were collected and transfected by a retroviral vector to express the neomycin resistance gene. These cells were then given back to the patients. The foreign gene served as a marker for the TILs and the transfected cells were shown to be present in tumors of all patients. Gene modified cells were also found in the circulation of the patients for as long as 64 days after administration. Three of the five patients experienced at least a partial response to the IL-2 - TIL therapy. No toxic effects from the TIL transductions were noted. Trials with transduced cells that bear the gene for tumor necrosis factor are planned and evaluations of TILs in human clinical trials continue.

The precise mechanism of TIL lysis of tumor is not yet established, but several clues indicate that tumor necrosis factor may be an important component. There are several reports in the literature which show that TIL express mRNA for TNF-α and TNF-β (103, 104). In addition, the level of mRNA expression for TNF and the antitumor activity of the TILs from human and ovarian cancers correlate (104-106). TNF, along with γ-IFN, has also been shown to increase target cell susceptibility to lysis by TILs. Finally, culturing the TILs with TNF in addition to the IL-2 improves the lytic activity of the lymphocytes (95).

A.6. IL-2 Alone As a Pharmacologic Agent

Numerous studies in rodents and humans have demonstrated that decreased immune responsiveness *in vitro* can be reversed by addition of IL-2. Examples include the generation or augmentation of immune functions in lymphocytes from nude mice (107-109) or aged animals (110); and overcoming immunosuppressive agents such as

cyclophosphamide (111). IL-2 has also been shown to be active *in vivo*. The earliest studies illustrating such an effect were conducted in nude mice (107, 109) and specially prepared rats (112) and demonstrated that IL-2 could reinstate an immune response or allograft rejection in immunocompromised animals. Similarly, an antibody against the IL-2 receptor was shown to prolong cardiac allograft survival in mice, indicating that IL-2 is important in graft rejection (113).

Experiments using animal models have shown that IL-2 alone can mediate tumor regression and clarified the mechanism for this activity (114-116). Administration of IL-2 preserves the lives of mice that had been implanted with tumor cells. The IL-2 was not effective if the animals were γ-irradiated or immunocompromised, but the action of the lymphokine was enhanced if the animals were immunized with tumor cells and lymphocytes from tumor bearing animals. Thus IL-2 requires involvement of the host immune system to be effective.

IL-2 alone has been used as an immunotherapeutic in patients with disseminated cancer. The first reported studies of IL-2 in humans gave to patients doses of IL-2 obtained from the supernatants of mitogen induced normal human lymphocytes (117). The amounts of IL-2 given ranged from 2-8 x 10^4 units and spontaneous lymphocyte proliferation and the development of atypical lymphocytes in circulation were reported, but no regression in tumor was seen. In another early trial (118), the source of the IL-2 was from the supernatants of Jurkat tumor cell cultures. The cells were cultured in serum free media with added phorbol ester. A total of 16 patients were tested, some receiving i.v. doses and some direct injection of the IL-2 into the tumor nodules. In this pilot study, the serum half life of the Jurkat IL-2 was determined to be approximately 5 to 7 minutes with a second phase of clearance of 30 to 120 minutes. The toxicity that was seen was dose related with no effects at 250 U/kg but at 2,500 U/kg chills, malaise, and fever were noted. At higher doses mild, reversible hepatic dysfunction was noted as well. No apparent regression of tumor was seen among the patients in this study.

A subsequent study took place when recombinant IL-2 became available (119). Again the clearance of IL-2 was very rapid and extremely high doses were necessary for detectable levels in the serum. When repetitive doses of 10,000 U/kg of IL-2 were given, the lymphokine was detectable in the peritoneum cavity, but not in the serum. At 100,000 U/kg doses, serum concentrations of 20 - 30 U/ml could be found. It was also found that IL-2 caused the very rapid clearance of LAK precursors from the peripheral blood of the patients. Other noteworthy findings include a transient increase in the level of γ-IFN in the patients, a 2 to 16 fold expansion in the total number of lymphoid cells were found at early time points after the IL-2 infusion had been stopped, and circulating soluble IL-2 receptor was found in the serum of patients shortly after the IL-2 administration (120). No patient treated with doses up to 3,000 U/kg/hr by continuous perfusion of IL-2 (for as long as 7 days) or with repetitive bolus injections of IL-2 (for 7 - 21 days) demonstrated significant tumor regression.

A trial where patients were treated with at least 30,000 U/kg three times a day with bolus injection was later published by the same group (121). Ten patients with a variety of malignant disorders were tested and treated intravenously or intraperitoneally. Three of six patients with melanoma experienced and objective regression, and there was no response in the other patients with colorectal (0/3) or ovarian (0/1) cancer.

In cases of non-disseminated cancer, IL-2 can be an effective reagent when it is administered locally. In a trial with patients who had malignant pleurisy due to lung cancer, 1,000 U/day of IL-2 was delivered into the pleural cavity by a catheter for 10 - 28 days. Pleural effusions and cancer cells in the perfusions disappeared within 10 days for 9 of the 11 patients in the study (122). In treating recurrent squamous cell carcinoma of the head and neck (123), patients received daily injections of 200 U of IL-2 for 10 days into the mastoid muscle region. Six of eight patients showed complete or partial regression following the therapy. The two patients who were non-responders had already undergone

functional or radical neck dissection. In both of the studies cited above, the toxicity of the therapy was judged not to be serious.

As has been mentioned before, IL-2 has associated toxicities when it is administered to patients in high doses (greater than 10,000 U/kg/day) (62, 63, 118-121, 124). In addition to the chills, fever, nausea, renal dysfunction, and malaise suffered by almost all patients receiving high doses of IL-2, IL-2 has cause pulmonary edema (125), thrombocytopenia (126), vascular leakage (127), dermatological inflamations (128), and necrosis or perforation of the colon (129). These side effects are costly: they limit the dose and the potential effectiveness of the IL-2 treatment; they impair the quality of life of the patient; and they require that the patient be hospitalized so that prompt medical intervention is available and greatly increase the financial cost of the treatment. In appraising IL-2 based therapies, it is necessary to balance the effectiveness of the therapy with the associated costs and toxicities.

A.7. IL-2 With Other Agents in Therapy

In addition to being used alone or with cell perfusions, IL-2 has also been used with other immunoreactive agents in therapeutic studies directed against tumors. Generally these factors are other lymphokines (130), but the use of antibodies and other reagents have also been investigated. The rationale for using combinations of factors with IL-2 is that the other agents might augment the activity of IL-2 *in vivo* against cancerous cells. This might take place in four different fashions: by direct cytotoxic or cytostatic effects; by immunomodulation; by induction of differentiation, and by enhancing recognition of tumor.

A.7.1. IL-2 and Other Cytokines

TNF and lymphotoxin are cytokines that have a direct cytotoxic or cytostatic effect on tumor cells. TNF activates phospholipases which lead to the generation of prostaglandins, leukotrienes, and free oxygen radicals that are toxic to the cell. TNF can also cause death of cells by apoptosis (131), yet clinical trials with TNF alone have not shown any significant antitumor response (132, 133). Interferons, on the other hand, have a cytostatic effect which inhibits growth of normal and transformed cells (134). Clinically, α-IFN has been used against hairy cell leukemia and response rates of 70% - 90% have been achieved (135). α-IFN has also been useful in B cell lymphomas, chronic myeloid leukemias, and bladder and renal cell carcinomas. Interferons can also exert antitumor effect by modifying the immune response. γ-IFN not only enhances LAK activity, it causes an increase of MHC surface antigens and thus renders the tumor more visible to immune effector cells. Cytokines may also be able to induce malignant myeloid precursor cells to differentiate to more chemo-responsive or non-proliferating mature cells. Treatment of myeloid leukemia cells with GM-CSF can increase the fraction of leukemic blasts in S phase and thus enhance cytoarabine mediated cytotoxicity (136). GM-CSF might also be able to induce tumoricidal activity in macrophages (137, 138). Enhancing the recognition of tumor can be achieved by antibodies that recognize specific antigens on tumor cells.

The combinational use IL-2 and α-IFN together has been the subject of a number of studies. Since α-IFN is an effective therapeutic agent in its own right and works via different mechanisms that IL-2, the potential for synergistic effects seems promising. These effects have been reported in some animal models (139, 140) however, the hoped for synergisms have yet to be seen in human trials or with human lymphocytes. An *in vitro* study that examined the enhancement of activated killing by lymphocytes from children with melanoma showed that IL-2 alone was as effective as IL-2 + α-IFN (141). In another report (142), 54 patients with metastatic melanoma were evaluated under a protocol where the patients received 3 x 10^6 U/m^2/day of IL-2 by continuous infusion for 4 days while being injected with 6 x 10^6/m^2/day of α-IFN on days 1 and 4. The cycle was repeated

every 2 weeks for up to 26 weeks. One patient achieved a complete response and 10 patients a partial response. Nineteen patients were stable and 24 showed progressive disease. These results were not superior to what could be achieved by IL-2 alone. In a more intense regiment from the same study, patients were given 3 daily injections of 4.5 x 10^6 U/m^2 of IL-2 and 3 x 10^6 U/m^2 of α-IFN for 5 days. This regimen was repeated at intervals of 3 weeks for a total of 3 cycles. Of nine patients, one had a completer response, four a partial response, three had stable disease, and one progressed. These results were encouraging, but the toxicity was severe, and treatment had to be stopped in five patients. The conclusion must be that IL-2 and α-IFN have not yet been shown to work together synergistically to any significant degree in a clinical setting.

A lack of synergism between IL-2 and α-IFN was shown in an animal study that used a weakly immunogenic sarcoma tumor as a model system, but here it was reported that adding TNFα to the protocol did result in synergistic regression of the tumor (143). Substantial improvements as measured by regression of tumor, prolongation of survival, and cure rates were seen when the three factors were used together compared to any combination of two of the cytokines or any cytokine alone. However, none of the factors were effective, alone or in combinations, against a nonimmunogenic sarcoma model. An examination of the effects of IL-2, TNF, and γ-IFN in a mouse model with pulmonary metastases of an immunogenic sarcoma tumor demonstrated that IL-2 and γ-IFN, and IL-2 and TNF were synergistic in their ability to regress tumor but that γ-IFN and TNF were not (144). All three together gave the best results and any of the factors alone were not effective. The combination of TNF and IL-2 has also been evaluated *in vitro* (145-147) and have been shown to be synergistic in these model systems.

An animal study has reported that the combination of β-IFN and IL-2 was more effective than either alone in inhibiting an adenocarcinoma tumor model (148), but a phase I study to assess the ability of β-IFN and IL-2 to regress tumor in humans has also been published (149). Twenty-six patients with advanced malignancy were given IL-2 by

continuous perfusion and β-IFN by 2 hour i.v. infusion for 5 days each week for 4 weeks. The maximum tolerated doses for the combined therapy were determined to be 1.1 x 10^6 U/m^2/day for IL-2 and 6 x 10^6 IU/m^2/day for β-IFN. No patients achieved complete or partial response to therapy in this study and all of the usual side effects of IL-2 therapy were manifest.

A.7.2. IL-2 with Antibodies and Drugs

In addition to being used with other lymphokines, the use of IL-2 in antibody based therapies has also been investigated. These therapies usually fall into one of two strategies (150). The first is to use the antibodies to target tumor as the IL-2 increases immune cell activities. This approach seeks to make the tumor more visible or accessible to the heightened immunity. The second strategy targets other immune cells as the antibody acts as an additional immune activation factor.

Dealing first with the latter strategy, there are published reports of generating activated killer cells from cultures that have high titers of IL-2 and anti-CD3 antibody (151). The CD3 molecule is a part of the T cell receptor complex and antibodies directed against this molecule have been shown to be mitogenic in culture (152-154). Using anti-CD3 antibody is thought to be a way to substitute for the specific antigen that is required for some T cell activations. Therefore, the use of anti-CD3 in IL-2 rich culture would cause the activation and expansion of $CD4^+$ and $CD8^+$ T cells in addition to the NK cell types which usually populate LAK culture. When cultured for 12 days with IL-2 and anti-CD3, murine splenocytes increased 100 - 4,000 fold in number instead of 6 - 20 fold for cultures incubated in IL-2 alone. Furthermore, only 5 million of these cells were able to mediate regression of tumor in a mouse model, where other studies using similar systems required 30 to 100 million cells to see comparable effects. Thus using the anti-CD3 in LAK generation cultures is doubly effective: it enhances efficiency in both the cell generation and

in the cell killing. The effector subsets responsible for the killing activities from this system have been identified and the activity was reported to be split evenly between $CD3^+$, $CD16^-$ cells and $CD3^-$, $CD16^+$ cells (155). CD16 is a natural killer cell marker, so the effector cell population has both the natural killer type cells found in conventional LAK culture and the antigen specific T cells, as expected. Another noteworthy finding from these studies is that when the anti-CD3 was present during the killing assays to determine cytolytic activity, the effector cells were much more potent in killing their tumor targets. Variations of this system have been used with tumor-draining lymph node cells from a mouse model that resulted in the expansion of tumor specific killer cells (156) and to expand isolated $CD4^+$ cells to generate killing activity among that subpopulation (157). It has also been shown that TNF increases the *in vitro* activity available from this system (158).

The other major use for antibodies in IL-2 related therapies is to use the antibodies to target tumor cells. IL-2 has been shown to improve antibody dependent cellular cytoxicity (ADCC) in *in vitro* culture (159). LAK cells have been shown to be 10 - 100 fold more effective in killing tumor cells that had been pretreated with specific antisera than they were in killing identical targets without antisera *in vitro* . The increased generation of ADCC *in vivo* by IL-2 administration has been shown in animal models (160-161). It has also been reported that the addition of IL-1 or TNF-α can enhance IL-2 induced ADCC, while α-IFN and γ-IFN do not have this effect. None of the above lymphokines can induce ADCC by themselves (162).

Tens or hundreds of clinical trials are progressing that use combined therapies of antitumor monoclonal antibodies, IL-2, and sometimes LAK cell perfusions. Although detailed studies have not yet been published, abstracts are available that indicate that high dose IL-2 plus antibody may be more effective than either component alone (163, 164). In one investigation of melanoma patients, 10 of 23 evaluable patients had a partial response

in an IL-2 plus anti-melanoma antibody protocol, whereas only 1 in 17 responded to IL-2 alone (165).

A derivation of the procedures of using antitumor antibodies plus IL-2 is to combine the two reagents into one. This strategy requires the fusion of IL-2 with an antitumor antibody and has been prepared and analyzed in *in vitro* studies (166, 167). The ability of a resting TIL cell line (660) to kill tumor was enhanced over using antibody alone or antibody plus soluble IL-2. The suggested mechanism for this augmentation of activity was that the IL-2 serves both as an activation factor for the tumoricidal cells and as a homing factor that selectively attaches the tumor with these cells expressing the IL-2R.

IL-2 has been used in other protocols that include combining the lymphokine with various other chemotherapeutic agents or adjuvants. There are several hundred abstracts that describe ongoing *in vivo* and *in vitro* investigations that use IL-2 with a multiplicity of combinations of drugs and formats (163, 164). Thus far no single protocol stands out. In a variation of this theme, IL-2 can be fused to a toxin and can be used as an immunosuppresive agent (168). This drug might be useful for suppressing graft vs. host disease and transplant rejection in organ transplant systems.

A.8. IL-2 in Therapy: Overview

More than fifteen years after its isolation, and ten years into detailed studies, IL-2 still has not established a widespread role in clinical therapy. This fact is disappointing in light of the rather astounding activities of IL-2 in *in vitro* and *in vivo* tumor models; but these model systems are quite different from the realities of cancerous disease in human patients.

In vitro systems illustrate potential activities that are possessed by cells, but there is not necessarily any correspondence between these potential activities and the activities manifested by cells *in vivo*. The animal model system studies demonstrate that tumoricidal activities of IL-2 can be effective *in vivo*, but these models consist of otherwise healthy

mice or rats that are administered tumor cells that are established and maintained *in vitro*. The animals did not spontaneously derive their tumors, as did the human patients, nor have they incubated the tumor for a period greater than 1 or 2 weeks. In addition, they have not undergone the primary chemotherapeutic or surgical regiments that patients in the IL-2 studies have experienced, nor is the treatment period drawn out over weeks or months. Thus there are extensive limitations about how well animal systems can model real cancer patients.

It must also be understood that cancer is a very heterogeneous family of diseases. Tumors differ according to the tissues from which they originate, their location in the body, their three dimensional structure and vascularization, their immunogenicity and antigen expression, and according to their advancement and age. IL-2 based therapies may be effective for some types of tumor, such as renal cell carcinomas or metastatic melanomas, and not for others. Additionally, IL-2 therapies may not be effective for tumors that have advanced beyond a certain point, or which are not sufficiently immunogenic.

Since IL-2 has not turned out to be a wonder drug, just give it to any patient and watch them recover, the effective use of IL-2 depends upon understanding the activities of the lymphokine and understanding the dynamics of cancer. The more we know about how immune cells can kill tumor, about how IL-2 invigorates the immune system and affects a patient, and about how tumor cells interact with, evade, and suppress the immune system; the more effective the use of IL-2 will be. We are only at the beginning of our understanding of these phenomenon, so we are only beginning to understand the uses and limitations of IL-2.

A.9. References

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APPENDIX B: IL-2 RECEPTOR EXPRESSION MODEL

The following is a sample of the programs used to simulate IL-2 receptor chain expression. This particular code was used to generated the simulation of cells incubating with immobilized IL-2 that is referred to as Simulation B in Chapter 4.

IMMOBILIZED IL-2 ITERATIONS-
THIS PROGRAM EVALUATES THE EXPRESSION LEVELS OF IL-2 RECEPTORS WHEN CTLL-2 CELLS ARE INCUBATED WITH IMMOBILIZED IL-2. THE ASSUMPTIONS FOR THIS PROTOCOL THAT DIFFER WITH THE SIMULATIONS FOR SOLUBLE IL-2 INCUBATIONS IS THAT THE INTERNALIZATION RATE FOR RECEPTORS BOUND TO IL-2 IS 0. THIS PROGRAM DOES NOT ASSUME SATURATION LEVELS OF IMMOBILIZED IL-2 BUT INSTEAD ASSUMES AN IL-2 EQUIVALENT CONCENTRATION OF .33 UNITS (10pM).

The following is a list of the definitions for the matrix components along with the values used:

a1=-(kaI+ka*[I])	=-(1.9 ee-4 sec-1+ .0000016 sec-1)
a3=k-H	= .002 sec-1
a4=k-a*	=.00033 sec-1
a6=k-b*H	= 2.5 ee-6 sec-1
a7=-kH	= -(2.0 ee-5 sec-1 or 2.0 ee-6)
a9=-kb*H	= -(1.0 ee-5 sec-1)
a10=kaS	= 1.4 sec-1 or 4 sec-1 or 7 sec-1 or 10 sec-1
b2=-(kbI+kb*[I])	=-(1.9 ee-4 sec-1+.000015 sec-1)
b3=k-H	= .002 sec -1
b5=k-b*	= .0015 sec-1
b6=k-a*H	= 4.0 ee-5 sec-1
b7=-kH	=-(2.0 ee-5 sec-1 or 2.0 ee-6)
b8=-ka*H	=-(3.5 ee-4 sec-1)
b10=kbS	= 3 sec-1 or 5 sec-1

c3=-(k-H+kH*[I]+kHI) =-(.002 sec-1 +.0015 sec-1 + 1.9 ee-4 sec-1)
c6=k-H* = 3.8 ee-5 sec-1
c7=kH = 2.0 ee-5 sec-1 or 2.0 ee-6
d1=ka*[I] =.0000016 sec-1
d4=-(ka*I+k-a*) =-(1.9 ee-4 sec-1 or 0 + .00033 sec-1)
d6=k-a*H = 4.0 ee-5 sec-1
d8=-ka*H =-(3.5 ee-4 sec-1)
e2=kb*[I] =.000015 sec-1
e5=-(kb*I+k-b*) =-(7.7 ee-4 sec-1 or 0 + .0015 sec-1)
e6=k-b*H = 2.5 ee-6 sec-1
e9=-kb*H =-(1.0 ee-5 sec-1)
f3=kH*[I] =.0015 sec-1
f6=-(kH*I+k-H*+k-a*H+k-b*H) =-(7.7 ee-4 sec-1 or 0 + 3.8 ee-5 sec-1+ 4.0 ee-5 sec-1 + 2.5 ee-6 sec-1)
f8=ka*H = 3.5 ee-4 sec-1
f9=kb*H = 1.0 ee-5 sec-1

The actual program starts in the line following this paragraph. M is the matrix of coeffiecients of rate constants, and the values for the rate constants are then defined. The variable vector is then established with initial guesses for the values. An iterative Do Loop is then established which takes the dot product of the coeffiecient matrix and the variable vector, which yield the changes in the variables for a 'step'. The variable vector is then remodified by these calculated changes and the process repeats. An 'If' statement is used to print the variable values for every 1800 iterations or 30 minutes of simulated time.

```
M=List[{a1,0,a3,a4,0,a6,a7,0,a9,a10},
    {0,b2,b3,0,b5,b6,b7,b8,0,b10},
    {0,0,c3,0,0,c6,c7,0,0,0},
    {d1,0,0,d4,0,d6,0,d8,0,0},
    {0,e2,0,0,e5,e6,0,0,e9,0},
    {0,0,f3,0,0,f6,0,f8,f9,0}];
mc=M 1;
```

```
a1=-(.00019+.0000016);
a3=.002;
a4=.00033;
a6=.0000025;
a7=-(.00002);
a9=-(.00001);
a10=1.4;

b2=-(.00019+.000015);
b3=.002;
b5=.0015;
b6=.00004;
b7=-(.00002);
b8=-(.00035);
b10=2;

c3=-(.002 +.0015+.00019);
c6=.000038;
c7=.00002;

d1=.0000016;
d4=-(.00033);
d6=.00004;
d8=-(.00035);

e2=.000015
e5=-(.0015);
e6=.0000025;
e9=-(.00001);

f3=.0015;
f6=-(.000038+.00004+.0000025);
f8=.00035;
f9=.00001;
```

```
Block[{var={alpha,beta,H,alphaI,betaI,HI,alphbet,
                alphIbet,alphbetI,1},dp},
                alpha=20000; beta=100; H=2000; alphaI=30; betaI=5; HI=3500;
                alphbet=2000000; alphIbet=3000; alphbetI=100000;

  Do[(dp=mc.var;
        dp = Append[dp,0];
   dp = Append[dp,0];
        dp = Append[dp,0];
        dp = Append[dp,0];
        var=dp+var;
        var[[7]]=var[[1]] var[[2]]; var[[8]]=var[[4]] var[[2]];
        var[[9]]=var[[1]] var[[5]];
        If[IntegerQ[i/1800],Print[var],Goto[QQ]]
        Label[QQ]),
   {i, 1, 43200, 1}
   ];

   ]
```